Dynamics of H4 K16 acetylation by the SAS-I complex in *Saccharomyces cerevisiae*

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium
(Dr. rer. nat.)

im Fach Biologie

eingereicht an der
Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

von

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Tag der mündlichen Prüfung: 17.09.2014

Abstract

The MYST histone acetyltransferase Sas2, the catalytic subunit of the SAS-I complex, acetylates histone H4 lysine 16 (H4 K16Ac), which prevents the spreading of SIR-mediated heterochromatin at telomeres and is involved in silencing at the *HM* loci and the rDNA locus in *Saccharomyces cerevisiae*. Sas2 interacts with the histone chaperones Asf1 and CAF-1, the latter of which is mainly active during DNA replication in S-phase, suggesting a cell-cycle dependent incorporation of Sas2-mediated H4 K16Ac on a genome-wide scale during S-phase. Here, we found that upon activation of Sas2, H4 K16Ac on bulk histone H4 increased during S-phase, but not G1-phase, in an Asf1- and CAF-1-dependent manner. Unexpectedly, H4 K16Ac was not incorporated into chromatin during S-phase following its acetylation, which suggested the existence of a nuclear pool of H4 K16Ac that was likely to be mediated by histone chaperones. In addition to its role in preventing SIR spreading into heterochromatin-adjacent regions, Sas2-mediated H4 K16Ac is suggested to have a genome-wide function, since in *sas2*Δ cells, H4 K16Ac is decreased at the majority of open reading frames (ORFs). One hypothesis is that Sas2-mediated H4 K16Ac might protect euchromatic genes from being spuriously silenced by SIR proteins, thereby maintaining genes amenable to transcription. Accordingly, H4 K16Ac is highly enriched at poorly transcribed genes, but not at highly expressed genes. In this study, we found that H4 K16Ac became enriched at *GAL* genes upon repression. Importantly, this gene repression required the histone chaperone Spt6, which is a key regulator of chromatin structure in the wake of RNA polymerase II. In *spt6-1004* cells, H4 K16Ac levels were higher at highly and poorly transcribed genes compared to wild type cells, whereas H4 occupancy was lower than in wild type. Collectively, the data suggested an indirect effect of Spt6 in that it regulates H4 K16Ac levels by the incorporation of K16-unacetylated histone H4 during transcription. In contrast, the absence of other histone chaperones (Asf1, CAF-1, HIR, Rtt106) had no effect on H4 K16Ac enrichment upon gene repression. The incorporation of Sas2-mediated H4 K16Ac during gene repression further suggested a function for this modification in euchromatin. However, we could show that H4 K16Ac was not necessary to prevent the erroneous binding of the SIR complex in euchromatic regions. The decrease of H4 K16Ac at ORFs in *sas2*Δ cells was due to the lack of Sas2 activity and not caused by an activity of the HDAC Sir2. Thus, Sas2-mediated H4 K16Ac prevents the binding of the SIR complex only at subtelomeric loci, but not on a genome-wide scale.

Zusammenfassung

Die MYST-Histonacetyltransferase Sas2 in *Saccharomyces cerevisiae* bildet die katalytische Untereinheit des SAS-I-Komplexes und acetyliert Histon H4 an Lysin 16 (H4 K16Ac), was die Ausbreitung des Heterochromatins an Telomeren verhindert und an der transkriptionellen Stilllegung der *HM*-Loci und des rDNA-Locus beteiligt ist. Sas2 interagiert mit den Histonchaperonen Asf1 und CAF-1. Da CAF-1 vorwiegend während der DNA-Replikation in der S-Phase aktiv ist, ergab sich die Hypothese, dass Sas2-katalysiertes H4 K16Ac genomweit während der S-Phase ins Chromatin eingebaut wird. In dieser Studie konnte gezeigt werden, dass durch die Aktivierung von Sas2 die Menge von H4 K16Ac in der S-Phase, jedoch nicht in der G1-Phase, anstieg, wobei dieser Anstieg eine Abhängigkeit von Asf1 und CAF-1 zeigte. Interessanterweise wurde dieses H4 K16Ac jedoch nicht ins Chromatin eingebaut. Dies deutete auf die Existenz eines möglicherweise von Histonchaperonen gebundenen H4 K16Ac-Pools hin. Neben der Verhinderung der Ausbreitung des SIR-Komplexes in subtelomerische Regionen hat Sas2-katalysiertes H4 K16Ac auch eine genomweite Funktion, da das H4 K16Ac-Niveau an der Mehrheit der offenen Leserahmen (ORFs) in *sas2*Δ Zellen vermindert ist. Eine Hypothese ist, dass H4 K16Ac euchromatische Gene vor transkriptioneller Stilllegung durch den SIR-Komplex schützt, wodurch diese Gene zugänglich für die Transkription bleiben. Entsprechend ist das H4 K16Ac-Niveau an schwach transkribierten Genen hoch und an stark transkribierten Genen niedrig. Wir konnten zeigen, dass sich H4 K16Ac an den *GAL*-Genen während deren Repression anreichert. Dieser Einbau von H4 K16Ac während der Genrepression ist abhängig vom Histonchaperon Spt6, welches die Chromatinstruktur hinter der transkribierenden RNA-Polymerase II reguliert. In *spt6-1004* Zellen war das H4 K16Ac-Niveau an stark sowie an schwach transkribierten Genen höher als in Wildtypzellen, während die H4-Menge an diesen Genen reduziert war. Insgesamt weisen die Daten auf einen indirekten Effekt von Spt6 hin, indem es das H4 K16Ac-Niveau durch den Einbau von K16-unacetyliertem H4 während der Transkription reguliert. Die Abwesenheit anderer Histonchaperone (Asf1, CAF-1, HIR, Rtt106) zeigte keinen Effekt auf die repressionsgekoppelte H4 K16Ac-Anreicherung. Der Einbau von H4 K16Ac während der Repression deutete weiterhin auf eine genomweite Funktion dieser Modifikation hin. Jedoch konnten wir zeigen, dass H4 K16Ac nicht notwendig ist, um die Bindung des SIR-Komplexes in euchromatischen Regionen zu verhindern, da das verminderte H4 K16Ac-Niveau an ORFs in *sas2*Δ Zellen auf das Fehlen von Sas2 und nicht auf Deacetylierung durch Sir2 zurückzuführen war. Daher verhindert Sas2-katalysiertes H4 K16Ac die SIR-Bindung nur an subtelomerischen Loci, jedoch nicht in genomweitem Maßstab.

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Abbreviations

Table 1. Abbreviations.

Ac	Acetylation (e.g. at H4 K16 = H4 K16Ac)
Asf1	Anti-silencing function 1
bp	Base pair
BSA	Bovine serum albumin
CAF-1	Chromatin assembly factor 1
ChIP	Chromatin immunoprecipitation
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
f. c.	Final concentration
FACS	Fluorescence-activated cell sorting
Gal	Galactose
Gb	Giga base pair
HAT	Histone acetyltransferase
kb	Kilo base pair
Mb	Mega base pair
MYST	Family of histone acetyltransferases, to which Sas2 belongs
NAD^+	Nicotinamide adenine dinucleotide
NDR	Nucleosome-depleted region
ORF	Open reading frame
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PMSF	Phenylmethylsulfonyl fluoride
qPCR	Quantitative real-time polymerase chain reaction
RNA Pol II	RNA Polymerase II
SAS-I	HAT complex containing Sas2, Sas4, Sas5
Sas2	Something about silencing 2, HAT that acetylates H4 K16
Sas2-td	Sas2 heat-inducible degron
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sir	Silent information regulator
TBS	Tris buffered saline

TBST	Tris buffered saline with Tween 20
TPCK	Tosyl phenylalanyl chloromethyl ketone
TSS	Transcription start site
UAS	Upstream activating sequence
YM	Yeast minimal medium
YP	Yeast peptone medium
YPD	Yeast peptone dextrose medium

Saccharomyces cerevisiae genes were named according to nomenclature conventions of the *Saccharomyces cerevisiae* genome database (SGD).

Amino acids were given in the single-letter code, e.g. K = lysine.

1 Introduction

1.1 Eukaryotic genomes and epigenetics

A main feature that distinguishes eukaryotic from prokaryotic cells is the presence of a cell nucleus, in which the carrier of genetic information, the deoxyribonucleic acid (DNA), is localized. The genetic information of an organism, the genome, consists of protein-coding sequences, the so-called genes, and non-coding sequences. Whole genome sequencing in many eukaryotic organisms so far has shown that genome size can range from 12.2 Mb in the unicellular baker's yeast *Saccharomyces cerevisiae* up to 150 Gb in the Japanese canopy plant (*Paris japonica*). Although genome size increases with the complexity of an organism, there is no direct correlation between the complexity and the number of genes encoded in its genome, but rather there is a higher proportion of non-coding and repetitive DNA in large genomes. For example, the 3.1-Gb human genome comprises about 97% of non-coding DNA (Alexander et al. 2010) while containing 20,687 protein-coding genes (according to the latest count) and another 11,224 DNA stretches that are classified as pseudogenes (Encode Project Consortium et al. 2012). In contrast, the 12.2 Mb of the *Saccharomyces cerevisiae* genome, which was the first eukaryotic genome to be completely sequenced (Goffeau et al. 1996), contains about 6300 genes and only 27% of non-coding DNA (Alexander et al. 2010).

One problem in packaging chromosomal DNA to fit a cell's nucleus is posed by the chemical nature of the DNA, which is strongly negatively charged. In order to achieve a compaction of the large DNA molecules, the DNA is associated with proteins, histones and non-histone proteins, as so-called chromatin. In this nucleoprotein complex, negative charges of the DNA molecules are shielded, which contributes to a compaction of the DNA. In contrast to earlier hypotheses, this packaging of DNA is not static. Rather, chromatin is very dynamic, thereby regulating the accessibility of the genetic information. Thus, chromatin introduces an additional level of gene regulation, called "epigenetic", which describes heritable changes in phenotype that are not due to changes in the DNA sequence itself.

1.2 Structural organization of the eukaryotic chromatin

The basic organizing unit of eukaryotic chromatin is the nucleosome, which, as the nucleosomal core particle, consists of an octamer of histones and 146 bp of DNA wrapped around it. Each histone octamer consists of two copies of the core histones H2A, H2B, H3 and H4 (Kornberg 1974; Luger et al. 1997). Histones are small and basic proteins, which are

highly conserved among species. They consist of a histone fold domain mediating the binding to DNA, the histone fold extension and a flexible, N-terminal tail that protrudes from the surface of the nucleosome (Luger and Richmond 1998b; Luger and Richmond 1998a). The canonical organization of the nucleosome can be modified by posttranslational modifications of the histones (Bannister and Kouzarides 2011), by the replacement of canonical histones with histone variants or by chromatin remodeling, thereby influencing the chromatin state. The nucleosomal structure is completed by the binding of the linker histone H1 at the entry- and exit-point of the DNA at the nucleosome core particle. The histone H1 in *S. cerevisiae* (Hho1) is structurally distinct from that of higher eukaryotes (Patterton et al. 1998), and its chromatin association is restricted to specific loci within the genome, e.g. the rDNA sequences (Freidkin and Katcoff 2001). The nucleosome is the basic repeating unit of eukaryotic chromatin, arranged like "beads on a string" with about 10 bp of linker DNA between the nucleosomes in *S. cerevisiae* (Wang et al. 2008). This 10 nm wide structure of chromatin has been proposed to be further compacted into the 30 nm chromatin fiber (Finch and Klug 1976; Widom and Klug 1985), whose structure is still discussed controversially (Maeshima et al. 2010). Furthermore, chromatin is condensed by non-histone proteins to form the metaphase chromosome.

Already very early, chromatin was cytologically distinguished into two forms, the less condensed euchromatin and the compact heterochromatin (Heitz 1928). Euchromatin is characterized by an open conformation and generally contains actively transcribed genes. Heterochromatin is highly ordered in nucleosomal arrays, is repressive to transcription and contains only a few genes. This condensed chromatin state can be further divided into facultative and constitutive heterochromatin, the latter of which is condensed throughout the cell cycle, contains a high density of repetitive DNA elements and is crucial for the maintenance of genome integrity. In contrast, facultative heterochromatin is also transcriptionally silent in some cell types, but retains the potential to interconvert between heterochromatin and euchromatin during developmental processes or environmental changes (Grewal and Jia 2007; Trojer and Reinberg 2007).

One key feature of heterochromatin is its ability to spread, thereby influencing gene expression in a region-specific, sequence-independent manner. This process of inactivating chromosomal domains is also referred to as silencing. Heterochromatin can form even along a whole chromosome, e.g. in dosage compensation that adjusts the dosage of sex chromosome-linked gene expression in the different sexes of a species. In female mammals, one X chromosome is coated by the noncoding Xist RNA, which induces chromosomal changes like

hypoacetylation and lysine methylation on histones that result in silencing of the whole chromosome (Okamoto and Heard 2009). The link between the conformational status of chromatin and the transcriptional status of a gene was already observed by H. J. Muller. When he X-ray-irradiated embryos of *Drosophila melanogaster*, he obtained adult flies that exhibited patterns of variegated gene expression in the fly eye, as evidenced by changes in eye color in a subset of cells (Muller 1930). This effect is caused by an X-ray-induced chromosomal inversion, which positions a euchromatic region containing the *white* gene adjacent to heterochromatin, whereby the euchromatic region is silenced due to the spreading of the adjacent heterochromatin. This phenomenon, termed position effect variegation (PEV), is not exclusively observed in *Drosophila*. For instance in *S. cerevisiae*, reporter genes that are inserted adjacent to telomeres are silenced, which is referred to as telomere position effect (Gottschling et al. 1990).

In metazoans, silencing is established and maintained by histone modifications and the subsequent binding of repressive proteins, among others e.g. methylation of lysine 9 of histone H3 (H3 K9me) and the binding of heterochromatin protein HP1 (Nakayama et al. 2001). Additionally, heterochromatin is also assembled by nucleation by RNA interference (RNAi) and DNA-binding factors, methylation of CpG islands or the incorporation of histone variants (Grewal and Jia 2007). The baker's yeast *S. cerevisiae* lacks both HP1 and the RNAi machinery. Here, heterochromatin is established and maintained at the three heterochromatic loci, the telomeres, the silent mating-type loci and the rDNA locus, by the SIR proteins, among which is the highly conserved histone deacetylase Sir2 (Moazed 2001), which is discussed below (1.5.1).

Another way to alter the chromatin state is by replacing canonical histones with histone variants, which differ from their canonical variant in a few amino acids up to half of the protein sequence (Malik and Henikoff 2003). Additionally, in contrast to canonical histones, histone variants are expressed throughout the cell cycle (Sarma and Reinberg 2005). H2A.Z, a variant of the canonical histone H2A, is found to be incorporated in the nucleosomes surrounding the nucleosome-depleted region (NDR) at promoters of inactive genes (Guillemette et al. 2005), keeping the promoters poised for transcription (Zhang et al. 2005). Furthermore, in *S. cerevisiae*, H2A.Z is enriched near telomeres, but is absent from telomeric heterochromatin, thereby preventing the spreading of heterochromatin into adjacent euchromatin (Meneghini et al. 2003). H3.3, a variant of canonical histone H3, is specifically enriched within actively transcribed genes (Ahmad and Henikoff 2002). Upon gene induction,

both H3 and H3.3 are displaced, followed by selective deposition of H3.3, thereby compensating for transcription-coupled nucleosomal displacement (Wirbelauer et al. 2005).
Nucleosomes are not simply packaging units of chromatin, but rather regulate the accessibility of the genetic information in all nuclear processes, e.g. in DNA replication, transcription and DNA repair. In order to ensure DNA accessibility, nucleosomal localization can be altered by ATP-dependent remodeling complexes, which, in addition to nucleosome sliding, also mediate histone eviction and exchange as well as nucleosome deposition (Mueller-Planitz et al. 2013). Collectively, nucleosome positioning together with alterations of the nucleosomal composition governs the accessibility of the genetic information in eukaryotes.

1.3 Histone acetylation and its modifying enzymes

Specific amino acid residues within the sequences of the histone proteins, especially at the N-terminal tails, but also within the globular domain, are targets of a wide variety of posttranslational modifications. Lysine and arginine residues can be methylated, in which a higher level of complexity is added by different forms of methylation: lysines can appear mono-, di- or trimethylated, whereas arginines can be found mono- or dimethylated in a symmetric or asymmetric fashion. Further modifications comprise the phosphorylation of serine and threonine residues, ubiquitylation and sumoylation of lysines, ADP ribosylation, deimination and proline isomerization (Kouzarides 2007).
Acetylation of lysine residues was the first histone modification to be discovered (Phillips 1963). Early studies showed an association of hyperacetylated histones with actively transcribed genes (Allfrey et al. 1964). Due to its chemical nature, acetylation neutralizes the positive charge of lysine residues, thereby weakening charge-dependent interactions between a histone and nucleosomal DNA, linker DNA or adjacent histones. Thus, the accessibility of DNA to the transcription machinery is increased. Each core histone has multiple sites at which modifications are found. However, specific combinations of modifications can be found at specific loci within the genome, meaning that most single histones are not modified at every site. In *Saccharomyces cerevisiae*, histone H4 can potentially be acetylated at four N-terminal lysine residues (K5, K8, K12 and K16). 12% of total H4 is not acetylated at these four lysines, whereas 36% of total H4 is acetylated at one lysine, 28% at two, 13% at three and 12% at four lysine residues (Smith et al. 2003). Most monoacetylated histone H4 is acetylated at K16, and 80% of all H4 molecules are acetylated at K16 (Clarke et al. 1993; Smith et al. 2003). The model of acetylation-mediated charge neutralization of lysines of

histones was further tested by expression analysis in yeast strains harboring all possible combinations of lysine-to-arginine mutations at positions 5, 8, 12 and 16 of histone H4 to mimic the positively charged, unacetylated state of lysines at these positions (Dion et al. 2005). It was hypothesized that if charge effects are responsible for transcription regulation by these lysine residues, then similar sets of genes should be deregulated by all four single lysine-to-arginine mutants as well as by mutants carrying combinations of these lysine-to-arginine mutations. Only the K16 mutation has specific transcriptional consequences independent of the mutational state of the other lysines, whereas single mutations of lysines 5, 8 or 12 and combinations of these showed similar effects. Thus, acetylation of histone H4 is interpreted by two mechanisms: a specific mechanism for lysine 16 and a nonspecific, cumulative mechanism for lysines 5, 8, and 12. The specific role of the acetylation of lysine 16 of histone H4 will be discussed later (1.3.3).

In addition to other known sites of acetylation of lysines of histone H3, acetylation of lysine 56 within the globular domain of H3 is exemplarily introduced here. Lysine 56 is acetylated on newly synthesized histone H3 and thus is mainly found in S-phase but also during DNA repair (Masumoto et al. 2005; Maas et al. 2006; Recht et al. 2006). In this context, chromatin assembly factors, which deposit newly synthesized histones onto DNA, have a higher affinity for K56-acetylated than for K56-unacetylated H3 (Li et al. 2008). Hence, H3 K56 is rapidly deacetylated in G2-phase to preserve genome integrity (Celic et al. 2006). In contrast to its role in nucleosome assembly, H3 K56 becomes acetylated when nucleosomes at gene promoters are disassembled by histone chaperones upon transcription activation (Williams et al. 2008). Thus, H3 K56Ac seems to be a necessary mark for the binding of histone H3 to chromatin assembly factors.

In addition to its function in weakening interactions between DNA and histones, lysine acetylation of histones also recruits effector proteins, e.g. by serving as a binding platform for proteins containing a bromodomain, e.g. Gcn5 or Rsc1. Some of these proteins possess enzymatic activities influencing chromatin structure. For example, the bromodomain of Gcn5, a subunit of the SAGA histone acetyltransferase complex, is essential for the recruitment of chromatin remodelers and thus for transcriptional activation (Syntichaki et al. 2000). Rsc1 is a component of the RSC chromatin remodeling complex that is required for the expression of several genes (Bungard et al. 2004). Other histone modifications recruit effector proteins carrying different binding domains, e.g. methylated residues are recognized by chromo-like domains or PHD domains, and phosphorylated histone residues can be bound by a domain in 14-3-3 proteins (Kouzarides 2007).

The abundance of histone modifications makes "crosstalk" between these very likely. On the one hand, modifications may compete for lysine residues because several different modifications are known for this amino acid. On the other hand, one modification can influence an adjacent modification directly or indirectly by influencing the binding of effector proteins (Kouzarides 2007).

So far, many enzymes catalyzing histone modifications as well as their elimination have been characterized. In addition to the enzymatic elimination, histone marks can also be removed by histone eviction, substitution with histone variants or, for example, by dilution during DNA replication. In the next two chapters, histone acetyltransferases (1.3.1) and histone deacetylases (1.3.2) will be introduced in more detail.

1.3.1 Histone acetyltransferases (HATs)

Histone acetylation is catalyzed by histone acetyltransferases (HATs), resulting in important regulatory effects on chromatin structure and assembly, and transcription. HATs mediate the transfer of an acetyl group from acetyl-coenzym A to the ε-amino group of a lysine residue of a histone, thereby neutralizing its positive charge (Zentner and Henikoff 2013). On the basis of their catalytic domains and other sequence similarities, the diverse HATs can be grouped into different families: 1) Gcn5 is the founding member of the Gcn5 *N*-acetyltransferases (GNATs), including Gcn5, PCAF, Elp3, Hat1, Hpa2 and Nut1, 2) the MYST HAT family, comprising the founding members **M**OZ, **Y**bf2 (Sas3), **S**as2 and **T**ip60 as well as Esa1, human and *Drosophila* MOF, and 3) the remaining HATs including p300/CBP (CREB-binding protein), Taf1 and others (Lee and Workman 2007).

Gcn5 as the founding member of the GNAT histone acetyltransferase family was the first HAT characterized in *Saccharomyces cerevisiae* (Brownell et al. 1996). As many other members of the GNAT family, Gcn5 belongs to multisubunit HAT complexes, where it functions as a coactivator in transcriptional regulation at subsets of genes by mediating interactions with transcription activators and general transcription factors, e.g. with the TATA-binding protein (TBP) (Grant et al. 1997; Lee and Young 2000). As the catalytic subunit of the SAGA complex, Gcn5 acetylates histone H3 at promoter-proximal nucleosomes at K9, 14, 18, 23 and 27 and additionally histone H2B at K11 and 16 (Suka et al. 2001; Carrozza et al. 2003). The promoter selectivity of the catalytic activity of the SAGA complex is mediated by specific subunits, e.g. Tra1 (Brown et al. 2001). Gcn5 is also a subunit of the ADA HAT complex, whose physiological function is less clear, although it is also related to transcriptional activation of a subset of genes.

Like members of the GNAT family, members of the MYST HAT family are also subunits of larger complexes that function in different nuclear contexts. The MYST HAT Sas2 will be discussed in more detail below (1.3.3). Esa1, the catalytic subunit of the NuA4 as well as the piccolo NuA4 complexes, functions in transcriptional activation of genes as well as in transcriptional silencing at the rDNA locus (Smith et al. 1998; Clarke et al. 2006). Esa1 targets histone H4 K5, 8, 12 and 16 (Clarke et al. 1999) and H2A.Z K14 (Millar et al. 2006) and is the only essential HAT in yeast. It has been proposed that the larger Esa1-containing complex (NuA4) is recruited to promoters and represents targeted activity, whereas the smaller complex (piccolo NuA4) functions globally (Millar and Grunstein 2006). Sas3, another MYST HAT and catalytic subunit of the NuA3 complex, functions in transcription elongation (John et al. 2000) but is also involved in transcriptional silencing (Reifsnyder et al. 1996).

Histone acetylation is a reversible, dynamic process. Deacetylation of histones is mediated by a class of enzymes referred to as histone deacetylases, which will be briefly discussed below.

1.3.2 Histone deacetylases (HDACs)

Lysine deacetylation at histones is catalyzed by histone deacetylases (HDACs), which thereby influence chromatin dynamics. HDACs are grouped into four classes. In *S. cerevisiae*, class I HDACs comprise Rpd3, Hos2 and Hos1. Rpd3 deacetylates lysine residues of all four core histones. In *S. cerevisiae*, Rpd3 is the catalytic subunit of two functionally distinct HDAC complexes: Rpd3L antagonizes Sir2-dependent heterochromatin propagation (Zhou et al. 2009), whereas Rpd3S interacts with Set2-methylated histones and contributes to transcriptional elongation (Carrozza et al. 2005; Keogh et al. 2005). In *S. cerevisiae*, class II HDACs are Hda1 and Hos3, which both show specificity for all four core histones (Rundlett et al. 1996; Carmen et al. 1999). Different yeast HDAC complexes of classes I and II affect transcription of distinct, partially overlapping sets of genes.

Class III HDACs depend on NAD^+ for the deacetylation reaction and are referred to as sirtuins, according to the founding member of the family, the yeast protein Sir2. Sir2 deacetylates H4 K16Ac and interacts with Sir3 and Sir4 to form the SIR complex, which characterizes heterochromatin in budding yeast (Moazed et al. 1997; Imai et al. 2000).

In higher eukaryotes, all three classes of HDACs contain more phylogenetically related members (Ekwall 2005). Additionally, there is also a class IV of HDACs comprising only one single enzyme, HDAC11, which shares characteristics of both class I and class II HDACs (Gao et al. 2002).

1.3.3 The SAS-I histone acetyltransferase complex in *Saccharomyces cerevisiae*

The histone acetyltransferase (HAT) Sas2 in *Saccharomyces cerevisiae* belongs to the family of MYST HATs (1.3.1). Originally, the *SAS2* gene was identified in a screen for enhancers of silencing defects in *sir1*Δ background (Reifsnyder et al. 1996). Upon deletion of *SAS2*, the silent mating-type locus *HML* is further derepressed in a *sir1*Δ background, and silencing at telomeres is completely lost. Furthermore, *SAS2* was identified in a screen for suppressors of silencing defects of a defective allele of the silent mating-type locus *HMR* (*HMR***a**-*e***). In this screen, a deletion of *SAS2* was found to suppress the silencing deficiency of the *HMR***a**-*e*** allele (Ehrenhofer-Murray et al. 1997). Thus, Sas2 surprisingly has opposite effects on silencing at telomeres and *HML* in contrast to silencing at *HMR*. Due to its influences on silencing, *SAS2* was given its name "something about silencing".

Sas2 is associated with Sas4 and Sas5 in the SAS-I histone acetyltransferase complex (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001). Both subunits, Sas4 and Sas5, are required for the HAT activity of Sas2 in this complex (Sutton et al. 2003), and Sas4 and Sas5 have identical effects on silencing as Sas2 (Xu et al. 1999a; Xu et al. 1999b). In the SAS-I complex, Sas4 is the central subunit, bridging the connection between Sas2 and Sas5 (Schaper et al. 2005). The main target of Sas2-mediated acetylation is lysine 16 of histone H4 (H4 K16), and, to a much lesser extent, H3 K14 (Sutton et al. 2003). In *S. cerevisiae*, Sas2-mediated acetylation of H4 K16 (H4 K16Ac) prevents the spreading of SIR-mediated heterochromatin into euchromatic regions. Thereby, Sas2-mediated H4 K16Ac functions as a boundary in maintaining euchromatic identity (Figure 1). Upon deletion of *SAS2*, which is not lethal, SIR proteins spread into telomere-distal, euchromatic regions, leading to the formation of heterochromatin and the repression of genes within these regions (Kimura et al. 2002; Suka et al. 2002). Additionally, further factors play important roles in the formation of a boundary between hetero- and euchromatic regions. For example, the histone variant H2A.Z is incorporated in euchromatic regions next to heterochromatin, thereby contributing to the heterochromatin-euchromatin boundary (Meneghini et al. 2003). However, Sas2-mediated H4 K16Ac is required for the incorporation of H2A.Z at these boundary loci (Shia et al. 2006). Moreover, the HDAC Rpd3L, of which a deletion is lethal in *sas2*Δ background, also contributes to the formation of the boundary (Ehrentraut et al. 2010).

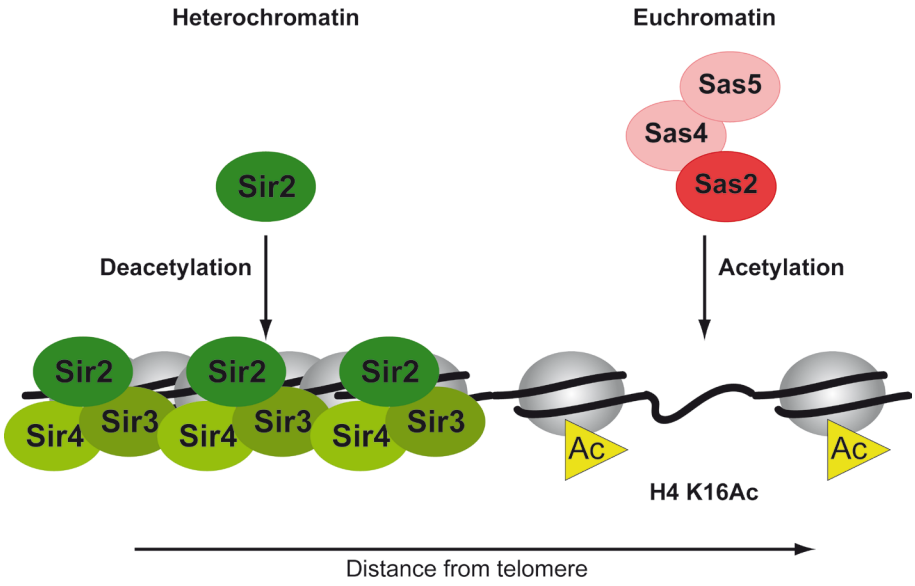

Figure 1. The histone acetyltransferase complex SAS-I prevents the spreading of heterochromatic SIR proteins by acetylating H4 K16 in euchromatic regions.
The SAS-I histone acetyltransferase complex with its catalytic subunit Sas2 acetylates histone H4 at lysine 16 (H4 K16Ac) in subtelomeric regions thereby preventing the excessive spreading of the heterochromatic SIR proteins (Sir2-4) from telomeres. Adapted from Kimura et al. (2002).

In addition to its function at heterochromatin-euchromatin boundaries, Sas2 also has a genome-wide function. Upon deletion of *SAS2*, H4 K16Ac is decreased at the majority of open reading frames (ORFs), whereas there is only little change in intergenic regions (Heise et al. 2012). Regions of low exchange of histone H3, for instance poorly transcribed genes, show the most pronounced loss of H4 K16Ac upon deletion of *SAS2*. This genome-wide function of Sas2-mediated H4 K16 acetylation is supported by the interaction of Sas2 with the chromatin assembly factors CAF-1 and Asf1 (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001). One hypothesis is that the SAS-I complex is recruited to newly synthesized DNA during replication via its interaction with CAF-1 and Asf1, which themselves interact with the sliding clamp PCNA at replication forks. Thereby, Sas2 is thought to be involved in the reestablishment of euchromatic patterns after DNA replication.

Furthermore, it was shown that removing H4 K16Ac is a necessary step in the condensation of metaphase chromosomes during mitosis. Aurora B-mediated phosphorylation of H3 S10 leads to the recruitment of the HDAC Hst2, which subsequently removes the acetyl group from H4 K16, thereby freeing the H4 tail to interact with the surface of neighboring nucleosomes and to promote fiber condensation (Wilkins et al. 2014). This finding challenges the hypothesis of the reestablishment of H4 K16Ac following DNA replication in S-phase and raises the question of how boundaries between euchromatin and heterochromatin are maintained in the time between mitosis and S-phase, in G1-phase.

In addition to its function in euchromatin maintenance, Sas2 has negative effects on longevity in *S. cerevisiae*. In replicatively old yeast cells, H4 K16Ac levels at telomeric loci are increased due to an age-dependent loss of Sir2 at these loci (Dang et al. 2009). Upon deletion of *SAS2*, senescence driven by a shortening of telomeres is delayed due to a stabilization of SIR proteins at the telomeres (Kozak et al. 2010). Thus, H4 K16Ac is involved in the regulation of cellular lifespan.

1.3.4 Functions of H4 K16 acetylation in other model organisms

Although all characterized H4 acetyltransferases can modify H4 K16, there are two HAT complexes that modify this residue for very specific functions – the SAS-I complex, introduced above (1.3.3), and the MSL (male-specific lethal) complex in flies and humans, which contains the Sas2 homolog MOF (Lee and Workman 2007). In addition to the MSL complex, MOF is also the catalytic subunit of the NSL complex (Mendjan et al. 2006).

In *Drosophila melanogaster*, the MSL complex is involved in the two-fold upregulation of the expression of X-chromosome-encoded genes in male flies (dosage compensation). Although other HATs in *Drosophila*, including the TIP60 complex, can acetylate H4 K16, the MSL complex with its catalytic subunit MOF carries out this specialized function on the X-chromosome (Akhtar and Becker 2000; Smith et al. 2000; Kind et al. 2008). The X-chromosome specificity of MSL in flies is provided by the MSL component MSL2, which is only expressed in males. Together with two functionally redundant RNAs, *roX1* and *roX2*, which associate with the MSL components, the MSL complex is targeted to the male X-chromosome (Ilik and Akhtar 2009). On the male X-chromosome, the MSL complex is not equally distributed. Instead, it is found peaking toward the 3' end of target genes (Alekseyenko et al. 2006; Gilfillan et al. 2006), where the MSL component MSL3 binds H3 K36me3, which is associated with active transcription. When H3 K36me3 levels are decreased by knock down of its catalyzing enzyme Set2, H4 K16Ac levels are also decreased (Larschan et al. 2007; Bell et al. 2008; Sural et al. 2008). MSL-mediated H4 K16Ac leads to a resistance against chromatin compaction and inhibits nucleosome remodeling (Shogren-Knaak et al. 2006). The decondensed chromatin caused by H4 K16Ac could thus facilitate increased transcription of the male X-chromosome. In contrast, Sun et al. (2013) propose that MSL-mediated H4 K16Ac does not mediate dosage compensation directly, but rather, that its activity overrides the high level of histone acetylation and counteracts the potential overexpression of X-linked genes to achieve the proper two-fold upregulation in males.

In addition to the MSL complex, in *Drosophila* there is another H4 K16Ac-catalyzing complex, the NSL complex, which also contains MOF as its catalytic subunit. In contrast to the MSL complex, the NSL complex is also active on autosomes, where it regulates the constitutive expression of housekeeping genes (Lam et al. 2012).

The human MSL complex also acetylates H4 K16, but in contrast to MSL in flies, it is responsible for the majority of genome-wide H4 K16Ac (Smith et al. 2005). Human MOF (hMOF), the catalytic subunit of the human MSL complex, predominantly binds promoters (Wang et al. 2009), where it likely functions in gene regulation. hMOF is an essential gene in mice, and its absence leads to early embryonic lethality and severe loss of H4K16 acetylation, indicating that hMOF is the major H4 K16 HAT in mammals (Gupta et al. 2008; Thomas et al. 2008). Notably, abnormal levels of hMOF, causing abnormal H4 K16Ac levels, correlate with malignant phenotypes (Laverty et al. 2010). hMOF not only acetylates histone substrates, but also non-histone proteins. For example, it acetylates p53, which can trigger apoptosis upon activation of the p53 target genes BAX and PUMA after induction of DNA damage (Sykes et al. 2006; Li et al. 2009). Both hMOF-mediated acetylation of histone as well as non-histone targets suggests that the regulation of these acetylation events are interesting targets for cancer therapy.

1.4 Chromatin dynamics

Eukaryotic chromatin is a highly dynamic complex of DNA associated with a wide variety of different proteins. These proteins, e.g. histones in the basic organizing unit of chromatin, the nucleosome, have a tremendous impact on all nuclear processes, e.g. DNA replication, repair and transcription. By modifications of the nucleosomal structure and position, as for instance by posttranslational histone modifications, replacement of canonical histones with histone variants or by histone sliding mediated by ATP-dependent chromatin remodelers, DNA accessibility is regulated. Nevertheless, the relatively stable association of the histone octamer and the DNA wrapped around it challenges processes requiring long-range access to the DNA, e.g. replication and transcription. Therefore, disassembly of nucleosomes to achieve DNA accessibility is an important process. Moreover, nucleosomes have to be reassembled, when DNA accessibility is no longer necessary, to tightly package the DNA and protect it from damage. Both processes, assembly as well as reassembly of nucleosomes, are mediated by chromatin assembly factors. These factors are also referred to as histones chaperones, since they bind and shield the positively charged histones to prevent their unspecific binding to the negatively charged DNA, which would be toxic for a cell.

1.4.1 Nucleosome assembly and histone chaperones

Histone chaperones mediate the assembly and disassembly of nucleosomes. Under physiological conditions, histones and DNA fail to self-assemble into nucleosomes because of the strong tendency of histones to associate non-specifically with the DNA and to thereby form toxic aggregates (Tyler 2002). The nucleosome is a modular assembly of stable heterodimers of histones H2A/H2B and H3/H4 associated with 146 bp of DNA (Ransom et al. 2010). The central 80 bp of the nucleosomal DNA is organized by a heterotetramer of H3/H4, whereas the peripheral 40 bp of DNA on each side are bound more loosely by two H2A/H2B dimers. H2A/H2B dimers are not assembled onto DNA until H3/H4 is deposited, and conversely, H2A/H2B dimers are removed from the DNA prior to the removal of H3/H4 (Figure 2). Nucleosome assembly is initiated by the assembly of H3/H4 dimers into H3/H4 tetramers. This might occur on the DNA via the sequential deposition of two H3/H4 dimers. Alternatively, H3/H4 tetramers could be formed on a histone chaperone before their deposition on the DNA, resulting in a tetrasome, an intermediate that has been observed in vitro and in vivo. Finally, the deposition of two H2A/H2B dimers gives rise to the nucleosomal core particle. Given that these H2A/H2B dimers do not physically interact with each other, both these dimers can be incorporated in a stepwise manner, giving rise to an intermediate structure with one H2A/H2B dimer, termed hexasome (Das et al. 2010). Due to this stepwise assembly and disassembly reaction, different histone chaperones for H2A/H2B (e.g. FACT, Chz1) and H3/H4 (e.g. Asf1, CAF-1, Rtt106, HIR, Spt6) have evolved to mediate these processes. Additionally, histone chaperones mediating the exchange of histone variants exist, e.g. SWR1 for the incorporation and Nap1 for the eviction of H2A.Z/H2B dimers, HIRA and Daxx for the exchange of H3.3/H4 (Das et al. 2010; Ransom et al. 2010).

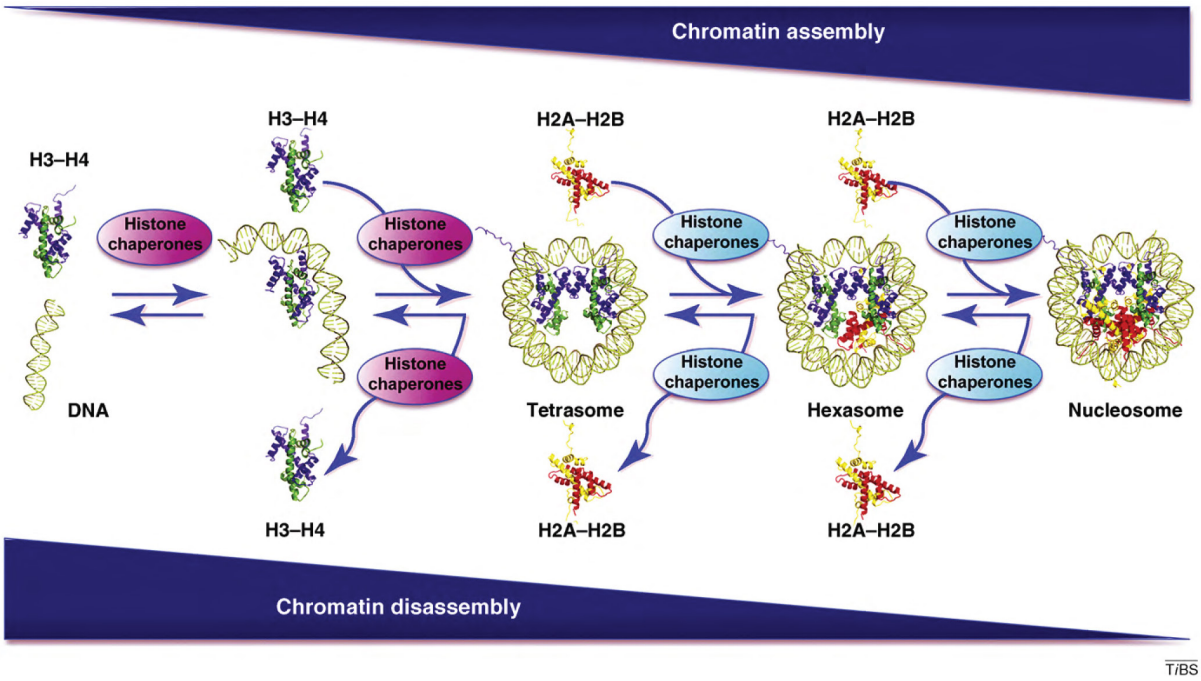

Figure 2. Histone chaperones mediate the stepwise assembly and disassembly of nucleosomes.
The assembly of the nucleosome, a complex of DNA wrapped around a tetramer of H3/H4 and two dimers of H2A/H2B is a stepwise process, in which each step is mediated by histone chaperones. Here, each step with possible intermediates is presented. A detailed description is given in the text. Histone H2A is depicted in yellow, H2B in red, H3 in blue, H4 in green. Taken from Das et al. (2010).

Although histone chaperones belong to diverse structural families with little sequence similarities, they share some common features. Many histone chaperones include a globular β-sheet core comprised of acidic patches for histone binding (Park and Luger 2008), and additionally, low complexity sequences that are rich in acidic amino acid residues (Hondele and Ladurner 2011). These flexible acidic tails might provide more than charge complementation of histones by mediating the transition of histones from chaperone to nucleosome and vice versa (Park et al. 2005). One well-studied example of histone chaperoning is the binding of H3/H4 dimers to the ubiquitous Anti-silencing factor 1 (Asf1). H3/H4 are bound to Asf1 as dimers, but in nucleosomes, H3/H4 is found as tetramer. This discrepancy is explained by the structure of Asf1 bound to an H3/H4 dimer (English et al. 2006). The edge of the hydrophobic β-sheet motif mediates the interaction with H3/H4 dimers while physically occluding the H3/H4 tetramerization interface. Thus, H3/H4 tetramers must be formed from two H3/H4 dimers, which can occur on the DNA or on a histone chaperone downstream of Asf1. Promising evidence for the latter hypothesis is given by crystal structures of a subunit of the chromatin assembly factor 1 (CAF-1), a histone chaperone acting downstream of Asf1. The p55 subunit of *Drosophila* CAF-1 (which corresponds to the Cac2 subunit of *S. cerevisiae* CAF-1) interacts with the α-helix 1 of histone H4 (Song et al. 2008), a region far away from the H3/H4 tetramerization interface. Apparently, Asf1 transfers

a H3/H4 dimer to the CAF-1 subunit Cac2, Asf1 dissociates subsequently, while a second H3/H4 dimer is transferred to Cac2 by Asf1, thereby forming a H3/H4 tetramer on a monomeric CAF-1 (Liu et al. 2012), which can then be deposited onto DNA.

In the next two sections, chromatin dynamics during DNA replication (1.4.2) and transcription (1.4.3) will be introduced, highlighting the functional roles of different histone chaperones during these processes.

1.4.2 Chromatin dynamics during DNA replication

Proliferating cells generate genetically identical daughter cells by cell division. One prerequisite for cell division is the faithful inheritance of the genetic as well as the epigenetic information to both daughter cells during the process of DNA replication in S-phase. Thus, not only DNA is duplicated during S-phase, but also the chromatin is the template for the replication machinery. In contrast to its template function, chromatin structure challenges DNA replication by restricting DNA accessibility. Hence, chromatin, i.e. also nucleosomes, have to be disassembled ahead of the replication fork, subsequently DNA is replicated and finally the chromatin is reassembled on the replicated DNA. Figure 3 schematically illustrates these chromatin dynamics coupled to DNA replication in S-phase.

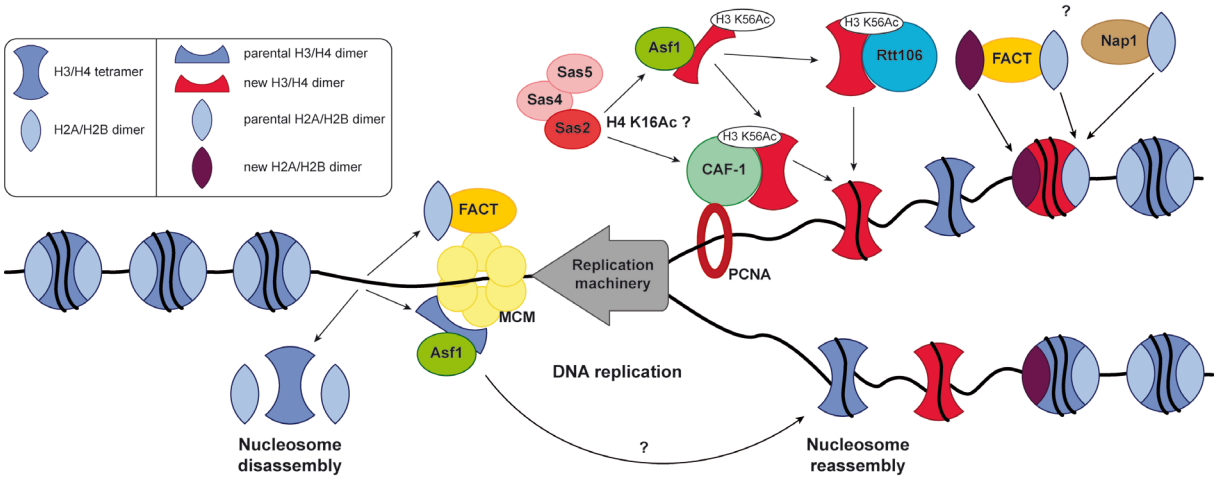

Figure 3. Chromatin dynamics during DNA replication in S-phase.
Nucleosomes are disassembled ahead of the replication fork to ensure DNA accessibility. The histone chaperone FACT is involved in H2A/H2B dimer disassembly, whereas Asf1 removes H3/H4 dimers. Nucleosomes are reassembled randomly from parental and newly synthesized histones on replicated DNA mediated by the histone chaperones Asf1, CAF-1 and Rtt106 for H3/H4, and most likely by FACT for H2A/H2B. A detailed description is given in the text. Adapted from Ransom et al. (2010).

As a first step in chromatin disassembly, the chromatin structure is opened by chromatin remodeling machineries prior to replication initiation at origins of replication (Ehrenhofer-Murray 2004). The helicase MCM2-7 (minichromosome maintenance) advances ahead of the

replication fork, thereby unwinding double-stranded DNA to facilitate the passage of DNA polymerases (Avvakumov et al. 2011). Simultaneously, the helicase MCM2-7 disrupts nucleosomes, releasing their component histones. Via an interaction with MCM4, the histone chaperone FACT (facilitates chromatin transcription) is recruited to evict H2A/H2B dimers (Gambus et al. 2006). Subsequently, the histone chaperone Asf1 is recruited, forming a MCM-H3/H4-Asf1 complex that disassembles an H3/H4 dimer (Groth et al. 2007), leaving the DNA accessible to be replicated by the progressing DNA polymerases. Such nucleosome-free or "naked" DNA stretches can constitute approximately 300 bp ahead and 250 bp or more behind the replication fork (Sogo et al. 1986; Gasser et al. 1996).

Following the passage of the replication fork, nucleosomes are reassembled on the replicated DNA by the incorporation of newly synthesized histones, which must make up at least half of the histones incorporated, as well as by the recycling of parental histones. The mechanisms for the transfer of parental and newly synthesized histones seem to be somewhat distinct. Nevertheless, there is no particular preference for the deposition of parental or newly synthesized histones either on the leading or the lagging strand. Thus, nucleosome reassembly is not semiconservative as DNA replication itself (Sogo et al. 1986; Jackson 1988).

Synthesis of canonical histones is tightly coupled to S-phase to provide sufficient amounts of histones to package the newly synthesized DNA (Hereford et al. 1981). Incorporation of newly synthesized histones H3 and H4 is mediated by the histone chaperones Asf1, CAF-1 and Rtt106 (regulator of Ty1 transposition 106). Newly synthesized H3/H4 dimers associate with Asf1, which is required for the acetylation of lysines 9 and 56 of histone H3 (H3 K9Ac, H3 K56Ac) by the histone acetyltransferase (HAT) Rtt109 (Recht et al. 2006; Driscoll et al. 2007; Han et al. 2007; Tsubota et al. 2007; Fillingham et al. 2008). Additionally, Asf1 recruits the HAT Hat1 to facilitate the acetylation of H4 K5 and K12 (Fillingham et al. 2008). Following these acetylations, H3/H4 dimers are handed over to the histone chaperones CAF-1 as well as Rtt106 to form H3/H4 tetramers, which are then deposited on the replicated DNA. CAF-1 is a highly-conserved, three subunit complex (Cac1, Cac2, Cac3 in *S. cerevisiae*) which assembles nucleosomes preferentially on replicated DNA (Smith and Stillman 1989). CAF-1 physically interacts with the sliding clamp PCNA (proliferating cell nuclear antigen) (Krude 1995; Shibahara and Stillman 1999; Rolef Ben-Shahar et al. 2009), which places CAF-1 immediately behind the progressing replication machinery. In contrast to H3 K9Ac and acetylation of H4, Asf1-Rtt109-mediated H3 K56Ac was shown to strongly enhance the affinity of CAF-1 as well as Rtt106 for H3 and to promote the incorporation of new histones into nucleosomes (Li et al. 2008). The histone chaperone Rtt106 is also implicated in

chromatin assembly on replicated DNA, because it interacts physically and functionally with CAF-1 (Huang et al. 2005). How CAF-1 and Rtt106 coordinate the assembly of H3/H4 tetramers on replicated DNA or whether chromatin assembly by these histone chaperones is redundant, remains to be determined. After a H3/H4 tetramer has been assembled onto the DNA, two H2A/H2B dimers are incorporated to form the nucleosomal core particle. Two histone chaperones, FACT and Nap1 (nucleosome assembly protein 1), may be involved in this process. However, it is still unclear whether one or both deposit H2A/H2B dimers onto the DNA (Ransom et al. 2010).

In addition to newly synthesized histones, histones derived from parental chromatin are also recycled and incorporated onto replicated DNA. So far, it is not clear which histone chaperones might be involved in this process. Additionally, it is also under debate, whether parental H3/H4 is incorporated as a tetramer, or whether parental H3/H4 tetramers are disrupted and H3/H4 dimers are then incorporated at their original locus on replicated DNA. The latter hypothesis would provide a mechanism for the reestablishment of epigenetic marks in a semiconservative manner, in that parental H3/H4 dimers would be the template for the establishment of epigenetic marks on newly synthesized and incorporated H3/H4 dimers. Nevertheless, there is strong evidence for the parental H3/H4 tetramer to be reassembled onto replicated DNA as a whole, and not as mixed tetramers consisting of one parental and one newly synthesized dimer (Prior et al. 1980; Jackson 1988).

However, a fundamental question of epigenetics remains: How are chromatin marks inherited to daughter cells during DNA replication. Acetylation marks associated with newly synthesized histones H3/H4 (H3 K9, K56, H4 K5, K12) are rapidly deacetylated after their chromatin incorporation (Annunziato and Seale 1983; Benson et al. 2006). For example, evidence related to the inheritance of Sas2-mediated acetylation of H4 K16 in *S. cerevisiae* comes from the observation that Sas2 physically interacts with the histone chaperones Asf1 as well as CAF-1 (Meijsing and Ehrenhofer-Murray 2001). This suggests that H4 K16Ac is reestablished at euchromatic loci in a replication-coupled manner. Furthermore, there are two possibilities for how the H4 K16Ac pattern can be reestablished: prior to assembly, or after the assembly of the histones onto the DNA. However, experimental proof for the replication-coupled incorporation of H4 K16Ac is missing.

1.4.3 Chromatin dynamics during transcription

In eukaryotic nuclei, protein-coding genes are transcribed into messenger RNA (mRNA) by RNA polymerase II (RNA Pol II). These mRNAs are the template for protein synthesis at

cytosolic ribosomes. Transcription is a stepwise process, initiated by the binding of activators upstream of the promoter (including the transcription start site (TSS)). This activator binding facilitates the binding of coactivators such as the HAT complex SAGA or the binding of mediators, e.g. chromatin remodelers, both of which lead to the binding of general transcription factors (Li et al. 2007a). By a combination of TFIID, TFIIA and TFIIB, RNA Pol II is positioned at the promoter, thereby forming the transcriptional preinitiation complex (PIC) in its closed form. Upon unwinding of the DNA at the TSS, the PIC is opened by positioning the single stranded DNA template in the RNA Pol II cleft, thereby initiating RNA synthesis. RNA Pol II escapes the promoter, transcribes 20-50 bases downstream of the TSS and pauses. This pausing is mediated by the negative elongation factor (NELF) and others. During this pausing step, RNA Pol II is phosphorylated at serine 5 at its C-terminal domain (CTD). To escape from the pause, RNA Pol II is phosphorylated at serine 2 in its CTD. This phosphorylation loosens the contacts between RNA Pol II and the general transcription factors, causing RNA Pol II to escape from pausing and to proceed to the elongation step of transcription. Having transcribed the gene, RNA Pol II is removed from the DNA, RNA is released, and the liberated RNA Pol II can reinitiate (Fuda et al. 2009).

Since transcription in eukaryotes does not occur on “naked” DNA, the chromatin packaging of the transcriptional template into nucleosomes appears to affect all stages of transcription and has to be overcome by RNA Pol II (Li et al. 2007a). In addition to the need for the disassembly of nucleosomes, several histone modifications have been related to active transcription, which themselves influence nucleosome remodeling, disassembly and reassembly. Markers for active transcription at the 5’ end of genes are for instance Set1-mediated H3 K4me3 (Santos-Rosa et al. 2002), H3 K9Ac, H3 K14Ac, H4 K5Ac and H4 K12Ac (Li et al. 2007a). A histone mark that is important for the restoration of chromatin structure in the wake of elongating RNA Pol II is Set2-mediated H3 K36me3. Set2 recognizes the elongating RNA Pol II due to its phosphorylation at serine 2 in the CTD. Thus, H3 K36me3 is high at 3’ ends of ORFs. Furthermore, H3 K36me3 recruits the HAT Rpd3S, which catalyzes histone deacetylation, thus keeping ORFs in a hypoacetylated state, which in turn inhibits spurious transcription from cryptic promoters (Carrozza et al. 2005; Joshi and Struhl 2005; Li et al. 2007b).

For proper transcription, nucleosomes have to be disassembled by evicting histones from the transcriptional template. Numerous activities are involved in this process of histone eviction. Cooperative binding of transcription factors, chromatin-remodeling complexes and the actively transcribing RNA Pol II itself contribute to histone displacement. The evicted

histones are bound by histone chaperones to prevent their rebinding to the DNA. Behind the progressing RNA Pol II, nucleosomes are reassembled by histone chaperones to repackage the DNA.

Transcriptionally inactive but inducible genes have a nucleosome-depleted region (NDR) at their promoter about 200 bp upstream of the start-codon (Yuan et al. 2005). This NDR is flanked by nucleosomes containing the histone variant H2A.Z, which keeps the gene poised for transcription (Guillemette et al. 2005; Zhang et al. 2005). Upon gene activation, activators are recruited to the promoter. This binding can occur on DNA packaged with nucleosomes (Adkins et al. 2004), but it is further stimulated by the activity of chromatin-remodeling complexes (Utley et al. 1997). Upon activator binding to the promoter, coactivators, such as chromatin-remodeling complexes (e.g. Swi/Snf), histone-modifying enzymes (e.g. SAGA) and mediators, are recruited to make the DNA accessible for general transcription factors (Li et al. 2007a). Thus, nucleosome depletion at promoters of active genes is a general property of eukaryotic genomes (Lee et al. 2004; Yuan et al. 2005). H2A.Z-containing nucleosomes flanking the NDR are resistant to chromatin remodeling (Li et al. 2005). Upon transcriptional activation, H2A.Z is acetylated by the SAGA complex and rapidly evicted from the promoter (Zhang et al. 2005), thereby making the promoter accessible for general transcription factors and RNA Pol II.

At the promoter as well as within the coding region, nucleosomes are disassembled ahead of the transcription machinery by the eviction of H2A/H2B dimers followed by the disassembly of H3/H4 tetramers. Figure 4 schematically illustrates the disassembly and reassembly of nucleosomes mediated by histone chaperones at the promoter and the coding region of genes. The histone chaperones Nap1 as well as FACT mediate the eviction and the reassembly of H2A/H2B dimers. Nap1 is able to cooperate with many other factors associated with transcriptionally active genes, as e.g. chromatin remodelers, to facilitate the disassembly (Lorch et al. 2006). In contrast, FACT directly affects transcription-coupled nucleosome disassembly. It tracks with the elongating RNA Pol II (Mason and Struhl 2003) and also interacts with many elongation-coupled factors, e.g. topoisomerases, Spt6 and RPA (Petesch and Lis 2012). Beyond nucleosome disassembly, FACT was also found to promote histone deposition on DNA (Belotserkovskaya et al. 2003). It is still not yet clear if Nap1 and FACT have redundant or separate functions in nucleosome disassembly, and further, whether one or both contribute to nucleosome reassembly in the wake of RNA Pol II passage.

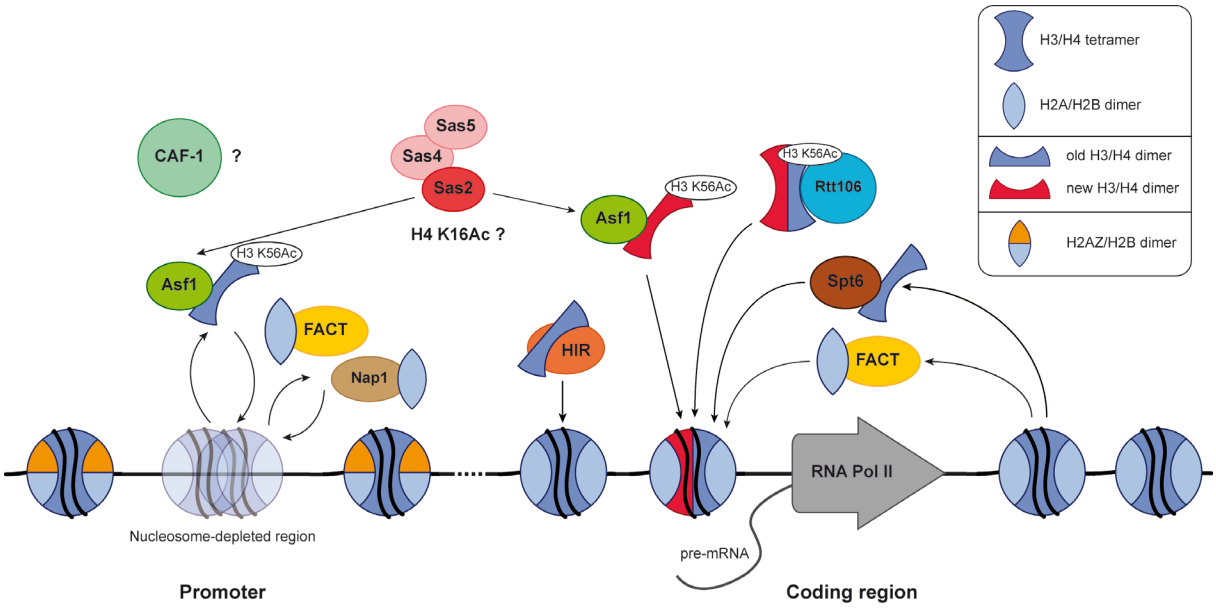

Figure 4. Histone chaperone mediated chromatin dynamics during transcription.
Nucleosomes constitute obstacles for the formation of the transcriptional preinitiation complex (PIC) at the promoter as well as for the progressing RNA polymerase II (RNA Pol II) at coding regions during transcriptional elongation. These nucleosomal barriers are overcome by the corporate activity of chromatin remodelers and histone chaperones. A detailed description is given in the text. Adapted from Avvakumov et al. (2011).

The histone chaperone Asf1 is involved in the disassembly as well as in the reassembly of H3/H4 tetramers during transcription initiation and elongation. Asf1 is recruited to promoters and coding regions of active genes (Schwabish and Struhl 2006). The activities of Asf1 during transcription might be linked to its regulatory role in Rtt109-mediated H3 K56 acetylation. H3 K56Ac, which peaks at promoters during transcriptional activation, is necessary for the recruitment of the Swi/Snf chromatin remodeler (Xu et al. 2005; Rufiange et al. 2007; Williams et al. 2008), which facilitates the establishment of a nucleosome-depleted region. Nucleosomes of activated promoters are highly dynamic, with a high rate of histone turnover (Dion et al. 2007), which suggests disassembly as well as reassembly events at the same promoter. Thus, during the first round of transcription, Asf1 might facilitate nucleosomal eviction from the promoter to allow for the assembly of the PIC. Thereby, Asf1 may incorporate the less stable H3 K56Ac-containing H3/H4-dimers into nucleosomes, which can be displaced more easily in further rounds of transcription initiation (Avvakumov et al. 2011).

The histone chaperone Spt6 (SuPpressor of Ty's 6) also plays a role in chromatin structure remodeling during transcription elongation (Bortvin and Winston 1996; Ivanovska et al. 2011). In an *spt6* mutant, nucleosome levels are greatly reduced over some coding regions, with nucleosome loss preferentially occurring over highly transcribed genes. Spt6 directly interacts with RNA Pol II (Yoh et al. 2007) and travels with its elongating form. Thus, high

levels of Spt6 can be found at actively transcribed genes. Mutations in both Spt6 and the Spt16 subunit of FACT cause spurious transcription from cryptic transcription start sites within the coding region of genes (Bortvin and Winston 1996; Hartzog et al. 1998; Kaplan et al. 2003) due to a low nucleosomal occupancy at these sites. Thus, FACT and Spt6 collaborate to disassemble nucleosomes ahead of RNA Pol II and restore chromatin structure behind it (Schwabish and Struhl 2004; Li et al. 2007a). Furthermore, Spt6 was found to play an important role in the deposition of di- and trimethyl groups on H3 K36 by the Set2 methyltransferase (Carrozza et al. 2005; Youdell et al. 2008), which recruits the HAT Rpd3S to transcribed regions, which in turn is necessary for histone deacetylation to recompact chromatin after transcription. In the fission yeast *Schizosaccharomyces pombe*, Spt6 is essential for the maintenance of K4 and K9 methylation of histone H3 in euchromatin and heterochromatin, respectively (DeGennaro et al. 2013; Kato et al. 2013). In addition to its role in transcription elongation, Spt6 also regulates the transcription level of a gene by controlling the position of the +1 nucleosome at the TSS (Ivanovska et al. 2011; Perales et al. 2013).

The histone chaperone Rtt106, which was shown to deposit H3/H4 tetramers on replicated DNA (1.4.2), is also active in nucleosome reassembly during transcription elongation. A deletion of *RTT106* shows genetic interaction with mutations in genes encoding transcription elongation factors, as e.g. Spt6 (Imbeault et al. 2008). Rtt106 is associated with coding regions of actively transcribed genes and, like Spt6, is required for the repression of spurious transcription from cryptic promoters by regulating the nucleosomal occupancy at coding regions (Imbeault et al. 2008; Silva et al. 2012). Whether Rtt106 is also involved in the disassembly of nucleosomes ahead of RNA Pol II, or whether it only contributes to the deposition of new histones that are marked by H3 K56Ac in the wake of RNA Pol II is not known yet.

The H3/H4 chaperone HIR (histone regulatory, HIRA in higher eukaryotes) is also involved in the control of transcription initiation as well as in transcription elongation. The HIR complex assembles nucleosomes that are resistant to the Swi/Snf remodeler (Prochasson et al. 2005) while recruiting the RSC remodeling complex (Ng et al. 2002) to the promoter, leading to transcriptional repression. A depletion of the Hir1 subunit of HIR leads to a delay in the reassembly of nucleosomes at promoters of different genes, while a loss of Spt6 completely abolishes it (Avvakumov et al. 2011). In higher eukaryotes, the HIRA chaperones incorporate the histone variant H3.3, which corresponds to canonical histone H3 in *S. cerevisiae*, within transcribed regions (Henikoff and Ahmad 2005).

So far, the histone chaperone CAF-1, which plays an important role in the assembly of nucleosomes on replicated DNA, has not been shown to be involved in transcription-coupled nucleosome assembly. Furthermore, it is not known whether acetylation of H4 K16, which is mediated by the SAS-I HAT complex, occurs during transcription. SAS-I can interact with Asf1 (Meijsing and Ehrenhofer-Murray 2001). Possibly, SAS-I could also interact with Asf1 during transcription-coupled reassembly of nucleosomes, thereby incorporating H4 acetylated at K16. However, experimental evidence for this hypothesis is missing.

1.5 Control of transcription and transcriptional repression

Transcription is the target of a wide variety of different regulatory mechanisms. A subset of genes, the so-called housekeeping genes, is more or less constitutively expressed due to the basic and essential cellular functions that their gene products fulfill. In contrast, the expression of most genes encoded within a genome is extensively regulated by intra- as well as extracellular signals during development, differentiation, cell-cycle progression and by environmental changes. In this section, three different examples of transcriptional regulation in *S. cerevisiae* will be introduced. Transcriptional silencing mediated by SIR-proteins, the regulation of the expression of genes necessary to metabolize galactose (*GAL* genes), and the cell cycle-dependent expression of canonical histone genes will be discussed.

1.5.1 SIR-mediated silencing

In *S. cerevisiae*, heterochromatin is restricted to three different genomic regions: The telomeres, the silent mating-type loci *HML* and *HMR* and the rDNA locus. In contrast to higher eukaryotes, heterochromatin in budding yeast is special in that it is not characterized by H3 K9 methylation and enrichment of HP1, but instead, silencing in *S. cerevisiae* is mediated by the SIR (silent information regulator) proteins Sir2, Sir3 and Sir4 (Kueng et al. 2013). Nevertheless, budding yeast heterochromatin shares features of heterochromatin in higher eukaryotes: histones are hypoacetylated, enzyme accessibility is reduced, late replication timing and sequestration at the nuclear envelope or near the nucleolus. Silencing in budding yeast is a three-step process: 1) nucleation involving the recruitment of SIR proteins by sequence-specific DNA-binding factors, 2) spreading of the SIR complex, and 3) termination of spreading by a boundary element or limited SIR protein supply. Upon binding of the Sir2/4 subcomplex to regulatory sites (silencers), the HDAC Sir2 deacetylates H4 K16 at adjacent nucleosomes. This deacetylation generates binding sites for Sir3 and Sir4 that

recruit additional Sir2. Thus, the Sir2/3/4 complex spreads along deacetylated chromatin (Rusche and Lynch 2009).

Telomeres, the ends of chromosomes, are one target of SIR-mediated silencing to stabilize and protect DNA ends. The yeast telomeric sequence consists of irregular repeats of $T(G_{1-3})$ with an average length of approximately 350 bp (Kupiec 2014). The subtelomeric regions are characterized by two other repeat families: X and Y' elements. X elements are present in all subtelomeric regions, whereas Y' elements, short TG repeats, are present in about half of the telomeres, located between the $T(G_{1-3})$ repeats and the X element. The $T(G_{1-3})$ repeats are nucleosome-free, whereas the subtelomeric repeats contain nucleosomes (Wright et al. 1992). To establish heterochromatin, Rap1 binds sequence-specifically to the $T(G_{1-3})$ repeats, subsequently recruiting Sir4 followed by Sir2 and Sir3 (Kueng et al. 2013). Thereby, the SIR complex spreads, which is antagonized by Sas2-mediated H4 K16Ac in adjacent euchromatic regions (Kimura et al. 2002; Suka et al. 2002).

Haploid *S. cerevisiae* cells exist in two different cell types, **a** or α, which are determined by the mating-type allele at the *MAT* locus (*MAT***a** or *MAT*α). At the *MAT* locus, transcription factors are encoded, either **a**1 and **a**2, or α1 and α2, that regulate genes that allow haploids to mate and form diploids. Additionally, cells contain two more loci, *HMR* and *HML* (homothallic mating-type right and left), encoding the **a** or α information, respectively (Kueng et al. 2013). At both these loci, silencing is necessary to maintain the mating ability of a cell. A derepression of the *HM* loci causes a coexpression of both **a**- and α-specific transcription factors. This coexpression also occurs in diploids and suppresses the mating program. The *HM* loci are flanked by silencers, *E* and *I*, to which the Sir proteins bind to mediate silencing (Brand et al. 1985).

The rDNA locus in *S. cerevisiae* comprises tandem array repeats, which are transcriptionally silenced in order to protect this locus from recombination, through which rDNA repeats would otherwise be excised as extrachromosomal circles (Gottlieb and Esposito 1989) leading to premature aging (Kaeberlein et al. 1999). Nevertheless, half of the genes at this locus are expressed. Repression within the rDNA locus uses a mechanism different to that at telomeres and the *HM* loci, which involves the RENT (regulator of nucleolar silencing and telophase) complex (Shou et al. 1999; Straight et al. 1999). In this RENT complex, Sir2 forms a complex with the nuclear protein Net1 and the phosphatase Cdc14.

1.5.2 The *GAL* regulon

In *S. cerevisiae*, glucose is the preferred compound to gain energy for all cellular functions by glycolysis. Nevertheless, if glucose is not available, other carbon sources can also be metabolized, as e.g. galactose. For this purpose, galactose has to be enzymatically converted into a compound that can be further metabolized in glycolysis, glucose-1-phosphate (Yarger et al. 1984). The enzymes required for the metabolism of galactose in the Leloir pathway (Reece 2000) are encoded by the *GAL* genes. When yeast cells are grown in the absence of galactose, these *GAL* genes are not expressed. If galactose is the only available carbon source, these genes become rapidly and highly expressed (Sellick and Reece 2005).

The *GAL* genes comprise two groups: the structural genes (*GAL1*, *GAL10*, *GAL7*, *GAL2*), which code for enzymes that catalyze the conversion of galactose into glucose-1-phosphate, and the regulatory genes (*GAL4*, *GAL80*, *GAL3*), which regulate the expression of the structural genes. The main activator of the expression of the *GAL* structural genes is Gal4, which is expressed in the presence of many carbon sources, e.g. raffinose or glycerol, but whose expression is severely reduced in the presence of glucose (Griggs and Johnston 1991). Gal4 is bound to the upstream activating sequence (UAS) in the promoter of the *GAL* structural genes. The activating activity of Gal4 is inhibited by an interaction with Gal80 in the absence of galactose (Platt and Reece 1998). Gal3 is a transcriptional inducer that adopts a conformation to interact with Gal80 in a galactose- and ATP-dependent manner (Zenke et al. 1996). The interaction of Gal3 and Gal80 in the presence of galactose abrogates the inhibition of Gal4, which leads to the recruitment of transcriptional activators, e.g. the HAT complex SAGA, RNA Pol II mediators and finally, the RNA Pol II holoenzyme itself, leading to transcription initiation at the *GAL* structural genes (Sellick and Reece 2005).

In the presence of glucose, the *GAL* genes are not only transcriptionally inert, but instead they are repressed. One main repressor of *GAL* gene expression in the presence of glucose is Mig1 (multicopy inhibitor of *GAL* gene expression 1). In the presence of glucose, cytosolic Mig1 becomes dephosphorylated by the Reg1-Glc7 phosphatase, which causes the import of Mig1 into the nucleus (Schuller 2003). There, Mig1 binds to the promoters of the *GAL* genes, thereby recruiting the Cyc8-Tup1 corepressor complex, which completely represses *GAL* gene expression. When cells become limited for glucose, Mig1 is phosphorylated by the Snf1 kinase complex and is exported to the cytosol.

Due to the well-studied regulation of the expression of the *GAL* genes, especially the *GAL* structural genes are a good model for investigations regarding transcription-coupled events during active transcription and repression.

1.5.3 Cell-cycle dependent control of histone gene expression in *S. cerevisiae*

The expression of canonical histones is highly cell-cycle dependent. During DNA replication, there is a high demand in new histones to package a genome, which has doubled in size. Therefore, to ensure an adequate histone supply during S-phase, histone expression in *S. cerevisiae* starts in late G1-phase (Hereford et al. 1981). In contrast, an excessive expression of histones outside of S-phase would lead to cytotoxic effects due to unspecific binding of the positively charge histones to the highly negatively charged DNA. Thus histone expression must be regulated in a cell-cycle dependent manner with a high expression during S-phase and only very low expression outside of S-phase (Kurat et al. 2014).

In *S. cerevisiae*, there are two copies of each core histone gene, each arranged in opposite orientation to a gene encoding its partner within the nucleosome: *HHT1-HHF1* and *HHT2-HHF2*, coding for H3 and H4, and *HTA1-HTB1* and *HTA2-HTB2*, coding for H2A and H2B. Each gene pair is regulated by centrally located, divergent promoter elements (Osley et al. 1986). Within the histone gene promoters, there are two *cis*-acting sequences regulating cell-cycle dependent histone gene expression: several copies of specific UAS sequences (Breeden 1988) and at three histone gene promoters (except *HTA2-HTB2*) a NEG sequence, which negatively influences histone gene expression (Osley et al. 1986). Additionally to these *cis*-acting sequences, there is a variety of *trans*-acting factors influencing histone gene expression. Among them, the histone chaperone HIR was the first to be identified as repressor of histone gene expression. HIR is recruited to the NEG sequence, where it is thought to assemble nucleosomes that occlude RNA Pol II recruitment, thereby repressing the histone expression (Kurat et al. 2014). HIR-mediated repression of histone transcription also involves the histone chaperones Asf1 and Rtt106. Together, HIR, Asf1 and Rtt106 mediate nucleosome assembly at NEG to repress histone gene expression. In order to repress the expression of *HHT1-HHF1*, *HHT2-HHF2* and *HTA1-HTB1*, HIR and Asf1 interact directly while the interaction with Rtt106 is mediated through histones H3/H4. Rtt106 recruits the chromatin remodeling complex RSC, which collaborates with Rtt106 to assemble H3/H4 onto chromatin, thereby occluding promoter sequences, which prevents the recruitment of RNA Pol II (Kurat et al. 2014). Additionally, the boundary element Yta7 (yeast tat-binding analog 7) is recruited to HIR-regulated histone gene loci (Zunder and Rine 2012), where it restricts Rtt106 to the regulatory region of NEG-regulated histone genes. To signal the repression of histone genes at the end of S-phase, a negative feedback seems to be in place, in which free histones, which are enriched at the end of S-phase, are bound by histone chaperones including

HIR, Asf1 and Rtt106, which in turn facilitates their assembly onto NEG-containing promoters with concomitant reduced recruitment of RNA Pol II (Kurat et al. 2014).

To activate S-phase-specific transcription of NEG-regulated histone genes, the HIR-Asf1-Rtt106 repression has to be overcome. So far, the following model could only be shown for the *HTA1* gene, but is also likely for the other NEG-regulated genes. Chromatin disassembly at these loci is enhanced by the incorporation of H3/H4 dimers, in which H3 is acetylated at K56 by the HAT Rtt109, thereby promoting a more "open" nucleosome structure. H3 K56Ac promotes Swi/Snf-dependent chromatin disassembly and subsequent activation of transcription (Kurat et al. 2014). The assembly of H3 K56Ac-containing H3/H4 dimers to histone promoters is likely to be mediated by UAS-recruited Spt10 and Spt21. How Spt10 and Spt21 are activated during late G1- and S-phase is currently not known. Also, the regulation, especially the cell-cycle dependent repression of the non-NEG histone genes *HTA2-HTB2*, is not clear. The only evidence for an activation of these genes is given by the recruitment of Spt10/21 to its promoters (Dollard et al. 1994).

1.6 Outline of this thesis

The MYST HAT Sas2, the catalytic subunit of the SAS-I complex that acetylates H4 K16, is involved in the maintenance of euchromatic identity next to heterochromatic loci in *Saccharomyces cerevisiae*. In addition, upon deletion of *SAS2*, H4 K16Ac levels are decreased on a genome-wide scale. In *sas2*Δ cells, H4 K16Ac is decreased at the center and the 3' end of ORFs, suggesting a genome-wide function of Sas2-mediated H4 K16Ac (Heise et al. 2012). Furthermore, it was shown that Sas2 interacts with the histone chaperones Asf1 and CAF-1 (Meijsing and Ehrenhofer-Murray 2001), the latter of which is mainly active in nucleosome assembly during DNA replication in S-phase. This led to the hypothesis that Sas2-mediated H4 K16Ac might be introduced into chromatin in a S-phase-dependent manner. Thus, one aim of this study was to characterize the cell-cycle dependence of Sas2-mediated acetylation of bulk H4 K16 and the cell-cycle dependent incorporation of H4 K16Ac into the chromatin. Additionally, we wanted to investigate the influence of Asf1 and CAF-1 on the rate of H4 K16 acetylation, since both these histone chaperones interact with Sas2. Upon activation of *SAS2*, we found, that H4 K16Ac on bulk H4 increased quickly during S-phase, but not during G1-phase. Unexpectedly, H4 K16Ac was not incorporated into chromatin on a genome-wide scale during the first S-phase after the activation of *SAS2*, but at genes that became repressed due to the experimental regimen. Furthermore, we wanted to

address whether H4 K16 acetylation is linked to histones synthesis, as is suggested by the observed S-phase-dependence of Sas2 activity.

One hypothesis for the genome-wide function of Sas2-mediated H4 K16Ac is that it might protect euchromatic regions from being spuriously bound by SIR proteins, which would lead to their silencing. In support of this hypothesis, upon glucose-induced repression of *GAL* genes, H4 K16Ac became enriched at these genes. In order to investigate the incorporation of H4 K16Ac into the chromatin upon gene repression, we asked which of the known histone chaperones (Asf1, CAF-1, HIR, Rtt106 and Spt6) is involved in the incorporation of H4 K16Ac outside of S-phase. For this purpose, cells with functional disruptions of these histone chaperones were tested by chromatin immunoprecipitation (ChIP) for their ability to incorporate H4 K16Ac during the repression of *GAL* genes. The incorporation of H4 K16Ac was only affected in cells that bear a mutation in Spt6, a key regulator of chromatin structure in the wake of RNA Pol II (Ivanovska et al. 2011). This effect of Spt6 on H4 K16Ac was found to be indirect in that it regulates the incorporation of K16-unacetylated histone H4.

Moreover, assuming that H4 K16Ac prevents spurious binding of SIR proteins in euchromatic regions on a genome-wide scale, we asked whether the decrease of H4 K16Ac at the center and the 3' end of ORFs in *sas2*Δ cells was due to the lack of Sas2 activity or caused by an activity of the HDAC Sir2 at these euchromatic loci. By chromatin immunoprecipitation combined with high-resolution tiling arrays (ChIP-chip), we found that the deletion of *SIR2* in *sas2*Δ background did not affect the decrease of H4 K16Ac at the center and the 3' end of ORFs in *sas2*Δ cells. Thus, H4 K16Ac as well as the available amount of SIR proteins might prevent euchromatic binding of the SIR complex only at subtelomeric regions.

In summery, this study confirms the cell-cycle dependence of Sas2-mediated H4 K16 acetylation and provides novel insights into the histone chaperone-regulated catalysis and chromatin incorporation of H4 K16Ac. The results of this study further support a function for Sas2-mediated H4 K16Ac in the maintenance of euchromatic identity by preventing SIR-mediated silencing at subtelomeric loci, but not on a genome-wide scale.

2 Material and methods

2.1 *Escherichia coli* strain

DH5α F$^-$ Φ80d *lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^- (Invitrogen)

2.2 Media and growth conditions

E. coli strains used for plasmid amplification were cultured according to standard procedures (Sambrook et al. 1989) at 37°C in Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin.

For the growth of *Saccharomyces cerevisiae*, media were prepared as described previously (Sherman 1991). Unless indicated otherwise, yeast was grown on full medium (YPD: 10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose). Full medium without carbon source (YP: 10 g/l yeast extract, 20 g/l peptone) was supplemented with 2% galactose (YP-Gal), 2% glucose (YP-Glu) or 2% raffinose (YP-Raff). Yeast minimal medium (YM: 6.7 g/l yeast nitrogen base w/o amino acids) was supplemented with 2% glucose or 2% galactose and as required with 20 µg/ml for adenine, uracil, tryptophan, methionine and histidine or 30 µg/ml leucine and lysine. Where applicable for selection, yeast cells were grown on medium supplemented with 200 mg/l geneticin to select for *KanMX* or on medium supplemented with 100 mg/l nourseothricine to select for *NatMX* or *natNT2*. For cell cycle arrest in G1-phase with α-factor mating pheromone, medium with pH 4.0 was used for cultivation of cells expressing the wild type Bar1 protease. For *bar1*Δ cells, the pH of the medium was not adjusted. Strains were grown at 30°C, unless indicated otherwise.

2.3 *Saccharomyces cerevisiae* strains

Yeast strains used in this study are given in Table 2. Yeasts were grown according to standard procedures (Sherman 1991). Yeast cells bearing the *spt6-1004* allele were grown at 37°C for at least 1.5 hours to inactivate Spt6.

Table 2. *Saccharomyces cerevisiae* strains used in this study.

Strain[a]	Genotype	Source[b]
AEY1	*MAT*α *ade2-101 his3-11,15 trp1-1 leu2-3,112 ura3-1 can1-100* (= W303-1B)	
AEY2	*MAT***a** *ade2-101 his3-11,15 trp1-1 leu2-3,112 ura3-1 can1-100* (= W303-1A)	
AEY3	*MAT*α *ADE2 lys2Δ his3-11,15 trp1-1 leu2-3,112 ura3-1 can1-100*	
AEY19	AEY1, *MATα ADE2 lys2Δ sir3Δ::HIS3*	
AEY264	*MAT***a** *his4*	
AEY265	*MAT*α *his4*	
AEY266	AEY2, *sas2Δ::TRP1*	
AEY269	AEY1, *sas2Δ::TRP1*	

AEY1558*	*MAT***a** *leu2 trp1 ura3-52 prc1-407 pep4-3 prb1-112*	
AEY2430	*MAT***a** *asf1Δ::kanMX*	
AEY2543	*MAT***a** *hir1Δ::kanMX ade2 LYS2*	
AEY2554*	AEY1558, *SIR2-9myc::TRP1*	
AEY2582	AEY1, *hmrΔ::URA3 sir2Δ::TRP1*	
AEY3461	AEY2, *cac1Δ::LEU2*	
AEY4148	AEY2, *natNT2-GALLpr-3HA-SAS2*	
AEY4488	*MAT***a** *ade2-101 his3-11,15 trp1-1 leu2-3,112 ura3-1 can1-100 ubr1Δ::GAL-*MYC*-UBR1::HIS3 sas2::Ub-Arg-DHFRts-HA-SAS2-URA3* (= *SAS2-td*)	
AEY4658	AEY19, *sas2Δ::TRP1*	
AEY4810	AEY4488, *hmlΔ::TRP1 cac1Δ::LEU2*	
AEY4812	AEY4488, *hmlΔ::TRP1 asf1Δ::kanMX*	
AEY5068	AEY2, *CAC1-9myc-natNT2*	
AEY5070	AEY2, *POL2-6HA-kanMX4*	
AEY5072	AEY2, *POL2-6HA-kanMX4 CAC1-9myc-natNT2*	
AEY5073	AEY2582, *sas2Δ::kanMX4*	
AEY5075	AEY2, *CDC45-6HA-kanMX4 CAC1-9myc-natNT2*	
AEY5092	AEY2, *CDC45-6HA-kanMX4*	
AEY5099	*MAT***a** *ade2-1 can1-100 his3-Δ200 leu2-3,112 trp1 ura3::LEU2 bar1::HIS3 Cdc45-*3FLAG-*kanMX* (MMY033)	(Vogelauer et al. 2002)
AEY5114	AEY5099, *CAC1-9myc-natNT2*	
AEY5120	*MAT***a** *ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1 can1-100 ybp1-1 RAD5*$^{+}$ *bar1Δ::HISg POL2-13MYC::TRP1* (AA119)	(Azvolinsky et al. 2009)
AEY5132	AEY5120, *CAC1-6HA-kanMX*	
AEY5236	*MAT*α *ADE2 lys2Δ* (W303) *natNT2-GALLpr-3HA-HHF1*	
AEY5250	*MAT***a** *natNT2-GALLpr-3HA-SAS2 natNT2-GALLpr-3HA-HHF1 ADE2 lys2Δ*	
AEY5258	AEY2, *bar1Δ::HIS3MX6*	
AEY5260	AEY266, *bar1Δ::HIS3MX6*	
AEY5262	AEY3461, *bar1Δ::HIS3MX6*	
AEY5264	AEY5250, *bar1Δ::HIS3MX6*	
AEY5281	AEY2430, *bar1Δ::HIS3MX6*	
AEY5289*	AEY2554, *sas2Δ::kanMX4*	
AEY5366	AEY3, *HHF2-6HA-kanMX4*	
AEY5370	AEY2543, *bar1Δ::HIS3MX6*	
AEY5380	AEY5258, *rtt106Δ::kanMX4*	
AEY5381	AEY2, *natNT2-GALLpr-HHF2-6HA-kanMX4*	
AEY5388*	*MAT***a** *his3Δ200 leu2Δ1 lys2-128Δ trp1Δ63 ura3-52 kanMX-GAL1pr-FLO8-HIS3 FLAG-spt6-1004*	
AEY5403	AEY2, *natNT2-GAL1pr-HHF2-6HA-kanMX4*	
AEY5410	AEY2, *RTT106-6HA-kanMX*4	
AEY5412*	AEY5388, *bar1Δ::URA3MX*	
AEY5421	AEY5410 + pAE240 (GPD_p-*SAS2*-PGK_t)	
AEY5432	AEY2 + pAE240 (GPD_p-*SAS2*-PGK_t)	

AEY5434 AEY266, *rtt106Δ::kanMX4*
AEY5436 AEY266, *RTT106-6HA-kanMX*4
AEY5467 AEY4148 + pAE 2105
AEY5471 AEY2, *RTT106-9myc-HIS3MX6*
AEY5486 AEY2, *SPT6-9myc-HIS3MX6*
AEY5487 AEY266, *SPT6-9myc-HIS3MX6*
AEY5495 AEY4488, *SAS2-td-6HA-natNT2*
AEY5499 AEY4812, *SAS2-td-6HA-natNT2*

[a]Unless indicated otherwise (*), strains were isogenic to W303. [b]Unless indictated otherwise, strains were constructed during the course of this study or were from the laboratory strain collection.

2.4 Genetic manipulation of *Saccharomyces cerevisiae* strains

Yeast strains used in this study are listed in Table 2. Unless indicated otherwise, yeast strains were either generated in this study by direct deletion, genomic integration, integration of plasmids, by crossing of yeast strains or they originated from the laboratory strain collection.

2.4.1 Crossing, sporulation and dissection of asci

Parental strains of different mating types were mixed in a drop of YPD medium and grown on a YPD plate over night at 30 °C. Diploids were isolated by streaking on selective YM plate. Sporulation was induced by plating the diploids on sporulation medium (19 g/l KAc, 0.675 mM ZnAc, 20 g/l agar) and incubating them for at least three days at 30 °C. For dissection of asci, cell walls were digested with zymolyase (5 mg/ml zymolyase, 1 M Sorbitol, 0.1 M Sodium citrate, 60 mM EDTA pH 8.0) for 6 min at room temperature, and the reaction was stopped by adding 100 µl water. The dissection of the digested ascospores was carried out using a micromanipulator (Narishige) connected to a Zeiss Axioscope FS microscope. The plates with the dissected ascospores were incubated for two to three days at 30°C. To follow the segregation of markers, plates were replica plated on selective medium.

2.4.2 DNA techniques in yeast

Gene deletions with *kanMX4* were performed as described before (Wach et al. 1994). This PCR-based knockout technique was also used for *HIS3MX6* and *URA3MX* knockouts, whereby the complete open reading frame of the targeted gene was replaced by the *HIS3MX6* or *URA3MX* sequence, respectively. N- and C-terminal tagging of proteins, as well as N-terminal insertions of *GALL*- or *GAL1*-promoters were performed using a PCR-based technique described in Janke et al. (2004). Oligonucleotides used for the amplification of deletion or insertion cassettes are listed in Table 4. The amplified cassettes were integrated into the yeast genome by homologous recombination. Correct deletion or integration was verified by PCR analysis.

2.5 Molecular cloning

Plasmid generation was performed according to standard cloning techniques (Sambrook et al. 1989). Plasmid isolation and gel elution kits were purchased from Qiagen and Macherey-Nagel. DNA polymerases used in PCR were purchased from Thermo Scientific (*Taq* polymerases) and from New England Biolabs (NEB) (Vent polymerase). Restriction endonucleases and respective buffers were purchased from NEB, T4 DNA ligase from Roche and the pGEM-T Easy vector system kit from Promega. For PCR-based gene deletions and insertions of N-terminal galactose-inducible promoters or N- or C-terminal tags, the respective cassettes were amplified from the appropriate plasmid by PCR. PCR products were transformed into yeast cells (Klebe et al. 1983). Deletion or integration was mediated by homologous recombination. For cloning of the *GAL1pr-HHF2-6HA* construct (AEY5403) into a 2µ plasmid (pRS423), the construct was amplified by PCR on genomic DNA (Hoffman and Winston 1987) inserting *Xho*I and *Eag*I restriction sites, subcloned in pGEM-T Easy and sequenced. The sequencing showed a silent mutation in *HHF2* and a V to G substitution in the linker between *HHF2* and the 6HA-tag. Because both mutations were not anticipated to perturb the function of Hhf2-6HA, the construct was cloned into pRS423 using the *Xho*I and *Eag*I restriction sites. Plasmids are listed in Table 3, oligonucleotides used for molecular cloning in Table 4. Oligonucleotides were purchased from Metabion, Martinsried.

Table 3. Plasmids used in this study.

Plasmid	Description	Source[a]
pAE240	YEp351-*GPD$_p$-SAS2-PGK$_t$*	
pAE267	pRS423	
pAE424	pAG60-*URA3MX*	
pAE478	pFA6a-*kanMX4*	
pAE929	pFA6a-*HIS3MX6*	
pAE1298	*natNT2-GALLpr-3HA* (pYM-N28)	(Janke et al. 2004)
pAE1809	*6HA-kanMX4* (pYM14)	(Janke et al. 2004)
pAE1884	*9myc-HIS3MX6* (pYM19)	(Janke et al. 2004)
pAE1995	*natNT2-GALLpr* (pYM-N27)	(Janke et al. 2004)
pAE2024	*natNT2-GAL1pr* (pYM-N23)	(Janke et al. 2004)
pAE2105	pRS423-*GAL1pr-HHF2-6HA*	
pAE2122	*6HA-natNT2* (pYM17)	(Janke et al. 2004)
pAE2154	*9myc-natNT2* (pYM21)	(Janke et al. 2004)

[a]Unless indicated otherwise, plasmids were constructed during the course of this study or were from the laboratory plasmid collection.

Table 4. Oligonucleotides used for molecular cloning and amplification of deletion and insertion cassettes.

Oligonucleotide	Sequence[a]
BAR1 S1	CTAAAATCATACCAAAATAAAAAGAGTGTCTAGAAG GGTCATATACGTACGCTGCAGGTCGAC
BAR1 S2	ATATTTGATATTTATATGCTATAAAGAAATTGTACTC CAGATTTCATCGATGAATTCGAGCTCG
BAR1 up	GCACGTCGAGCCTTGTCATG

RTT106 S1	GCTGACCAAAAAGCCACTATTCAAACGTAAAGTCCT ACAGATTTGCGTACGCTGCAGGTCGAC
RTT106 S2 KO	CTTACATATGCGTATTCATGCTATATTATAATATCGA ATCTAAGATCGATGAATTCGAGCTCG
RTT106 up	GAATTCAATTAGACATCATAAG
SAS2 S1	TATTGGAGGCTCCTATTTTCTAGTTGCTTTTTGTTTTC ACTCGCAAAAAAAACGTACGCTGCAGGTC
SAS2 S2 KO	TCGAGCGATATTCTATCCTGAAATACATATGCCATTA AGTTACATCCTGAATAGATTCATCGATGAATT
SAS2 up	CCATATCGAACTGGATAGAG
KanMX K2	GCCCCTGAGCTGCGCACGTC
CAC1 S2	CAGTTTATCTGTATGTTTCTATATACTAAAGATCCGT TCAAGTTAATCGATGAATTCGAGCTCG
CAC1 S3	TGGGAGAATCTTCGAGCCAATGCAAATATGCCAACC CCGTCTTTGCGTACGCTGCAGGTCGAC
POL2 S2	TTTTCATGGTAAAGAGGCCATTGAACCTCGCGTTATA TACTGCTTACTCAATCGATGAATTCGAGCTCG
POL2 S3	AGTATTACGGTTTTGATATATTATTGAGTTGTATTGC TGATTTGACCATACGTACGCTGCAGGTCGAC
CDC45 S2	TATATTCATATGCTGGTATATATGTACGACTAAATAA TATAAATTTGATTAATCGATGAATTCGAGCTCG
CDC45 S3	GTGAAGATCTTTCACCATTCCTGGAGAAGCTGACCTT GAGTGGATTGTTACGTACGCTGCAGGTCGAC
HHF1 S1	GTACTAAAGCAACAAACAAAAACAAGCAACAAATA TAATATAGTAAAATATGCGTACGCTGCAGGTCGAC
HHF1 S2	CGAATCCCAAATATTTGCTTGTTGTTACCGTTTTCTT AGAATTAGCTAAATTAATCGATGAATTCGAGCTCG
HHF1 S3	GTTGTTTATGCTTTGAAGAGACAAGGTAGAACCTTAT ACGGTTTCGGTGGTCGTACGCTGCAGGTCGAC
HHF1 S4	CTTGGCACCACCTTTACCTAGACCTTTACCACCTTTA CCTCTACCGGACATCGATGAATTCTCTGTCG
HHF1 up	CGTTCTGAAAACTTCGCATC
HHF1 down	GCTTGTTGTTACCGTTTTCTTAG
HHF1-2 mid	CTGGTTTGATCTACGAAGAAG
HHF2 S1	CCTACATCTTGTTCAAAAGAGTAGCAAAAACAACAA TCAATACAATAAAATAATGCGTACGCTGCAGGTCGA C
HHF2 S2	GAAAGGCATGAAAATAATTTCAAACACCGATTGTTT AACCACCGATTGTTTAATCGATGAATTCGAGCTCG
HHF2 S3	GATGTTGTTTATGCTTTGAAGAGACAAGGTAGAACC TTATATGGTTTCGGTGGTCGTACGCTGCAGGTCGAC
HHF2 S4	GTGACGCTTGGCACCACCTTTTCCTAGACCTTTACCA CCTTTACCTCTACCGGACATCGATGAATTCTCTGTCG
HHF2 up	CATTTGTATGGCAGGACGTTC
HHF2 down	CAAACACCGATTGTTTAACCAC

RTT106 S2 Tag	CTTACATATGCGTATTCATGCTATATTATAATATCGA ATCTAAGTCAATCGATGAATTCGAGCTCG
RTT106 S3	GATAATGATGACGATGAAGATGATGAGGATGGATCC GGAGTAGAATACGATCGTACGCTGCAGGTCGAC
SAS2 S2 Tag	GATATTCTATCCTGAAATACATATGCCATTAAGTTAC ATCCTGAATAGATTCCTAATCGATGAATTCGAGCTCG
SAS2 S3	GAACAAAACGGAATTGGTTCAAATTAAAAGATGAAT ATTTGCTGATAGATGACCGTACGCTGCAGGTCGAC
SPT6 S2	GGTCAAAGTAATAATAAAATTAATAATAACAATGGA CACTACATACGCATCTAATCGATGAATTCGAGCTCG
SPT6 S3	GACGCTTCTAAAATCTAACAGTAGTAAGAATAGAAT GAACAACTACCGTCGTACGCTGCAGGTCGAC
SPT6 3' end fwd	CTGGTAAACTACAATTATCCAAG
GAL1pr-HHF2-6HA fwd	GGGCTCGAGGAGCTCTAGTACGGATTAGAAGCCG
GAL1pr-HHF2-6HA rev	CCCCGGCCGCTTTTATATTTCTCTACAGGGGCGC

[a]Underlined parts of the sequences bind to plasmid sequence for cassette amplification.

2.6 Synchronization of *Saccharomyces cerevisiae* cells and FACS analysis

Strains bearing the heat-inducible *SAS2* degron allele (*SAS2-td*) were freshly grown on YP-Gal plates and used to inoculate a liquid culture of YP-Gal, at repressive conditions (37°C) during the day. An over night culture was inoculated to a final OD of 0.05. Cells were grown to OD 0.5 until the next day, harvested, washed with YP-Gal pH 4.0 and subsequently suspended in YP-Gal pH 4.0 containing nocodazole (f. c. 10 µg/ml) to arrest the cells in G2/M-phase. Cells were grown 2 to 3 h at 37°C. To control the cell cycle arrest, a small cell sample was sonicated 3 times for 250 ms, setting "high" (Bioruptor, Diagenode) and inspected under the microscope for large-budded cells. The M-phase-arrested cells were harvested, washed with YP-Gal pH 4.0 and subsequently suspended in YP-Gal pH 4.0 containing the mating pheromone α-factor (f. c. 1.62 µg/ml) to arrest the cells in G1-phase. At pH 4.0, the Bar1 protease, which digests α-factor, is inhibited. Cells were grown 1.5 h at 37°C and cell cycle arrest was verified under the microscope. Sample "0" was taken and processed for Western blot (20 OD) or chromatin immunoprecipitation (50-200 OD). The residual culture was divided into two parts. One half was maintained in α-factor and shifted to YPD / YP-Glu and 30°C to activate Sas2-td. The other half was also shifted to YPD / YP-Glu and 30°C to activate Sas2-td, but was released into S-phase by the addition of pronase (f. c. 30 µg/ml). Samples for Western blot or ChIP were taken at specific time points.

Cells bearing a deletion of *BAR1* were arrested in G1-phase by incubation in medium without adjusted pH adding α-factor to a final concentration of 25 ng/ml (no pre-arrest with nocodazole is necessary). To release these G1-arrested *bar1*Δ cells into the following S-phase, cells were washed with the respective medium and suspended in medium containing pronase (f. c. 100 µg/ml).

To determine the cell cycle stage in which the cells are arrest, and to follow cell cycle progression, small samples of cells were taken to stain their nuclear DNA using propidium iodid and measure the staining intensity by fluorescence-activated cell sorting (FACS). For

this purpose, 0.1 OD of cells were harvested, washed with water and fixed in 70% ethanol at least over night at 4°C. The fixed cells were then washed twice with 20xTE (0.2 M Tris pH 8.0, 0.02 M EDTA pH 8.0) and incubated with RNase A in 20xTE at a final concentration of 1 µg/µl for 4 hours at 37°C. Subsequently, the cells were washed twice with PBS (137 mM sodium chloride, 2,7 mM potassium chloride, 12 mM phosphate as hydrogen phosphate and dihydrogen phosphate) and stained with propidium iodide in PBS (f. c. 100 µg/ml) at 4°C over night. DNA content was measured and analyzed at a BD FACSCalibur or at a BD FACSAria II.

2.7 Antibodies

Antibodies used in this study are summarized in Table 5.

Table 5. Antibodies used in this study.

Antibody	Company / Source	Catalog and lot #	Application and notes
α-H4 K16Ac	Active Motif	39167 lot 01609002 lot 01008001 lot 33311003	ChIP: 4 µl per IP, Protein G Agarose
α-H4 K16Ac	Millipore	07-329 several lots	Western blot: 1:1000, 3% milk in TBST (0.05% Tween), 4°C over night
α-H4	Abcam	ab7311 several lots	Western blot: 1:1000, 3% milk in TBST (0.1% Tween)
α-H4	Millipore	05-858 several lots	ChIP: 4 µl per IP, Protein G Agarose
α-H2B	Active Motif	39237 several lots	Western blot: 1:3000, 5% milk in TBST (0.1% Tween), 4°C over night
α-HA	Covance	MMS-101P several lots	Western blot: 1:1000, 5% milk in TBST (0.1% Tween), 4°C over night ChIP: 5 µl per IP, Protein G Agarose CoIP: 4 µl per IP, Protein G Agarose
α-myc	Sigma-Aldrich	M4439 several lots	Western blot: 1:5000, 3% milk in TBST (0.1% Tween), 4°C over night ChIP: 5 µl per IP, Protein G Agarose
α-Sas2	Meijsing and Ehrenhofer-Murray (2001)	laboratory supply	Western blot: 1:1000, 3% milk in TBST (0.1% Tween), 4°C over night CoIP: 20 µl per IP, Protein A Sepharose
α-Actin	Abcam	ab8224 several lots	Western blot: 1:1000, 3% milk in TBST (0.1% Tween), 4°C over night
α-rabbit-HRP	Sigma-Aldrich	A0545 several lots	Western blot: 1:5000, in solution depending on primary antibody, 1h room temperatur

α-mouse-HRP	Sigma-Aldrich	A9044 several lots	Western blot: 1:1000, in solution depending on primary antibody, 1h room temperatur
α-guinea pig-HRP	Sigma-Aldrich	A7289	Western blot: 1:5000, in solution depending on primary antibody, 1h room temperatur

2.8 Preparation of protein extracts from *Saccharomyces cerevisiae*

For whole cell extracts, cells were grown in liquid culture to an OD of approximately 0.7-0.9. 20 OD of cells were harvested by centrifugation and washed with PBS containing protease inhibitors (PBS+PI; 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml TPCK, 40 µM PMSF). The cell pellets were stored at -80°C until protein preparation. To extract the proteins, cells were resuspended in 200 µl PBS+PI. Cells were broken with acid-washed glass beads by vortexing seven times for 30 s. 50 µl 4x Lämmli buffer (f. c. 62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) was added, mixed and boiled at 95°C for 10 min (for histone preparations) or 5 min (for other proteins). The extracts were then centrifuged for 5 min at 7000 rpm and the supernatant was recovered. The remaining pellet was washed again with 100 µl 1x Lämmli buffer, centrifuged and the supernatants were pooled. Protein extracts were stored at -80°C until further use in SDS-PAGE and Western blot. Before the extracts were loaded onto a SDS-polyacrylamide gel, they were incubated at 95°C for 5 min.

2.9 SDS-PAGE and Western blot

SDS-PAGE in Tris-glycine buffer according to standard methods (Laemmli 1970) was used for protein separation. Acrylamide concentration in the SDS-polyacrylamide gels was chosen according to the size of the protein to be detected. Transfer to nitrocellulose membranes (Amersham Hybond ECL, GE Healthcare) was accomplished by blotting with the BIO-RAD Tank Transfer System with 5.5 mA h cm^{-2}. For all proteins, except histones, transfer buffer with 39 mM Glycine, 48 mM Tris, 0.037% SDS and 20% methanol was used (Sambrook et al. 1989). For blotting of histones, transfer buffer with 25 mM Tris, 52 mM glycine, 20% methanol was used. The blot membrane was subsequently blocked for 1 hour at room temperature in 3% milk in TBST (50 mM Tris/HCl pH 7.5, 150 mM sodium chloride, 0.1% Tween 20, 3% skim milk powder), if not stated otherwise (2.7). After the incubation of the membrane with the primary antibody in the appropriate solution (Table 5) at 4°C over night, the membrane was washed shortly in TBST and incubated with the respective secondary antibody in the appropriate solution for 1 hour at room temperature. Subsequently, the membrane was washed in TBST several times for up to 2 hours. For signal detection, Amersham ECL Western Blotting Analysis System (GE Healthcare) and Amersham Hyperfilm ECL chemiluminescence films (GE Healthcare) were used.

2.10 Immunoprecipitation experiments

2.10.1 Coimmunoprecipitation (CoIP) of Rtt106-6HA and Sas2

For coimmunoprecipitation experiments of Rtt106-6HA and Sas2, 400 OD of cells of an exponentially growing culture of the respective strains were harvested by centrifugation, washed twice with water, resuspended in 5 ml lysis buffer (50 mM Tris pH 8.0, 150 mM sodium chloride, 1% NP-40, 1 tablet of Roche complete protease inhibitors per 50 ml), divided into 1 ml aliquots and frozen at -80°C until further use. To lyse the cells, acid washed glass beads were added, the cells were vortexed 10 min at 4°C and centrifuged at 5000 g for 15 min. The supernatant was split into aliquots of 100 OD. Per IP, one aliquot corresponding to 100 OD was used. For IP, the cell lysates were pre-cleared with 80 µl of Protein G agarose (for α-HA) or Protein A sepharose (for α-Sas2) rotating for 1 hour at 4°C. After centrifugation (3000 rpm, 30 s), the indicated amounts of antibodies (Table 5) were added to the supernatant, which was then incubated over night at 4°C. The next day, 160 µl of Protein G agarose or Protein A sepharose were added and incubated for 5 hours at 4°C, respectively. After centrifugation (3000 rpm, 30 s), the Protein G agarose or Protein A sepharose was washed three times with lysis buffer, the agarose or sepharose was suspended in 100 µl lysis buffer with 1x Lämmli buffer, incubated at 95°C for 5 min and appropriate amounts were loaded onto an SDS-polyacrylamide gel. Lämmli buffer was also added to the untreated input cell lysates, which were then also loaded onto the SDS-polyacrylamide gel in appropriate amounts.

2.10.2 Immunoprecipiation (IP) to enrich for tagged histone H4

For the immunoprecipitation of the 6HA-tagged histone H4, the same protocol as described for CoIP experiments (2.10.1) was used. Additionally, the IP method described in Nourani et al. (2001) was tested. Because in SDS-PAGE HHF2-6HA has a similar size as the light chains of the α-HA and α-H4 antibodies used, the antibodies were crosslinked to the Protein G agarose beads to minimize the release of light antibody chains from the beads. For this purpose, Protein G agarose beads were briefly washed twice with PBS and then over night rotating at 4°C. The beads were collected by centrifugation (14000 rpm, 1 min). Dilution buffer (PBS with 1 mg/ml BSA) was added in a ratio of 1:1 and rotated for 10 min at 4°C. The beads were collected by centrifugation and the supernatant was discarded. The antibody solution was prepared in dilution buffer in an appropriate concentration and added to the Protein G agarose beads at 1:1 ratio. The mixture was incubated rotating 1 hour at 4°C. Subsequently, the beads were collected by centrifugation, and the beads were washed with dilution buffer and PBS at 1:1 ratio, rotating for 5 min at 4°C each. For crosslinking of the bead-bound antibodies, 1 ml of a freshly prepared 13 mg/ml stock of dimethyl pimelimidate (DMP) was dissolved with 1 ml wash buffer (0.2 M triethanolamine in PBS). This DMP solution was added to the beads at 1:1 ratio and rotated at room temperature for 30 min. The pH of DMP was tested to be between 8 and 9 before and after addition to the beads. After the first crosslinking step, the beads were washed with wash buffer (5 min at room temperature). Subsequently, DMP solution was added for a second time at 1:1 ratio for 30 min rotating at room temperature. After washing the beads with PBS, the DMP crosslinking was repeated. To

quench the crosslinking, quench buffer (50 mM ethanolamine in PBS) was added at 1:1 ratio and rotated for 5 min at room temperature. After collecting the beads by centrifugation, the quenching was repeated once. The beads were washed with PBS, and the excess of unlinked antibodies was removed by repeated washing with 1 M glycine (pH 3.0) rotating for 10 min at room temperature. Finally, the beads were washed with the buffer used for IP and stored in this buffer until further use.

2.10.3 Chromatin immunoprecipitation (ChIP)

ChIP was carried out essentially as described in Weber et al. (2008), with the following exceptions. For the subsequent use of antibodies specific for histone H4 or acetylated H4 K16 and for ChIP of Sir2-9myc, crosslinking of 100 OD of yeast cells in 1% formaldehyde was performed for 30 min at room temperature. For Rtt106-9myc and Spt6-9myc, crosslinking was performed for 20 min, for Pol2-6HA and Cdc45-6HA, crosslinking was performed for 15 or 5 min at room temperature. Samples were sonicated at 4°C, seven cycles 30 s on and 60 s off, setting "high" (Bioruptor, Diagenode). 4 µl antibody was used for ChIP, unless stated otherwise (Table 5). RNase (10 mg/ml) digestion was carried out 1 hour at 37°C prior to incubation with proteinase K. DNA clean-up was performed using the Qiaquick Gel Extraction Kit and ERC cDNA Binding Buffer or QG Buffer mixed with isopropanol in a 3:1 ratio (Qiagen). DNA precipitates for ChIP-chip were eluted from the Qiaquick binding columns with distilled water. Quantitative real-time PCR (qPCR) for the analysis of ChIP samples was performed as described (Weber et al. 2008), except that SYBR Green Real MasterMix (5 PRIME) or PerfeCTa SYBR Green SuperMix (Quanta) was used (2.11). Oligonucleotides used for amplification are listed in Table 6.

2.11 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed on Rotor Gene 3000 (Corbett Research) or on RotorGene Q 2Plex HRM (Qiagen). The qPCR reactions were prepared with SYBR Green Real MasterMix (5 PRIME) or PerfeCTa SYBR Green SuperMix (Quanta) according to the manufacturer's instructions. Cycling was performed according to the manufacturer's instructions. qPCR was started by an initial denaturation step of 2 min at 95°C followed by 45 cycles of 15 s at 95°C, 30 s at 56°C and 45 s at 72°C. After 1 min at 50°C, melting curve detection was performed ramping from 50°C to 95°C every 5 s. The C_t value for each reaction was determined, and a standard curve of input samples was used to calculate the amounts of DNA precipitated during the ChIP experiment relative to input DNA. Three technical replicates were performed for every ChIP. The amount of DNA precipitated with the α-H4 K16Ac antibody was calculated relative to the amount of DNA precipitated with the α-H4 antibody at the respective regions. For most experiments, mean ChIP values and standard deviations of three independent biological replicates are given, unless stated otherwise. In some cases (as indicated), ChIP values are normalized to DNA precipitated at the *ACT1* control gene. Oligonucleotides used for qPCR amplification are given in Table 6.

Table 6. Oligonucleotides for qPCR used in this study.

Oligonucleotide	Sequence
ACT1 up (2)	GGTGATGGTGTTACTCACGTCG
ACT2	CAGCAGTGGTGGAGAAAGAG
ARS305 up (2)	CGCCGTAATAACTACTTTCGACAGAC
ARS305 down	GCATTCAAACTGGCCGGTCTTGC
ARS305+8kb up (2)	GACTCGGAAAGACCTTCAAGG
ARS305+8kb down (2)	GACAAATATCCCTGAGGTCAAAC
ARS305+17kb up (2)	GAATTCTGCCTTTGAATTTAACGC
ARS305+17kb down (3)	GTCGTTTCTGCGGTTTCAACG
ARS306-8kb up	GGGAGTCAATACCGTGTTCAGC
ARS306-8kb down	CGCAGAAAAAGTTGGCGCTTCC
ARS306 up	GCAAGCATCTTGTTTGTAACGCG
ARS306 down	CATGAAGTAATGATACCTCAGCG
ARS607 up	GCTTTGTCTTGTTTATATTTAGTTACGTTG
ARS607 down	GTGTCGCAGTCCATAGAAGGAG
ARS607+5kb up	CCCGAACGTTTGTTGAAATCTTCC
ARS607+5kb down (2)	GATTTACTAAATGCTATTTATCCCAAATAATC
ARS607+10kb up	GAGAGTCGTTTTTGTTGATTACTTTGC
ARS607+10kb down (2)	TGAACGCCTATCAGTTGTGTATTTC
CSF1 (0) fwd	CTTCTATTGACGGTAATAAGTTAGCAAGC
CSF1 (0) rev	GATTTTCGCTCGTTTCCATAGTAGCC
CSF1 (4.4) fwd	GACTTTCCAAAGGGCATGTGCG
CSF1 (4.4) rev	GCCGTATATATACGACCTTGCAACATC
CSF1 (8.8) fwd	CGATCCCAGGTAGCAAATATTTCC
CSF1 (8.8) rev	GCTTTTGGACGGGGATAACTGC
GAL3 5’ fwd	ATGAATACAAACGTTCCAATATTCAG
GAL3 5’ rev	GGCTAATGGCAAAACTGAAAAATC
GAL10 5’ fwd	GAAAATTCAATATAAATGACAGCTCAG
GAL10 5’ rev	CCTTTTCCAGACCTTTTCGGTC
GAL10 center fwd	GACGGCTAAACCAGATAGGGCC
GAL10 center rev	CTGGTGCCGGCACCAATAGTCAC
GAL10 3’ fwd	CAAGGCTTTTCATCCCGATTCC
GAL10 3’ rev	GAAGGATAGTAAGCTGGCAAATC
RIP1 fwd	CAACCGTAGAAACCTTTATTTCATC
RIP1 rev	GTCCTTCAAAGCGGACATATCC
SSA1 5’ fwd	GTAAATAACAGATAATATGTCAAAAGCTG
SSA1 5’ rev	GGTCGTTGAAGTTTCTACCGATC
SSA1 3’ fwd	GACACCGTCACCAAGAAGGCTG
SSA1 3’ rev	GCCGCACCAATTGGCTTAATCAAC
Tel VI R 0.5 up (2)	CGAGTGGATGCACAGTTCAGAG
Tel VI R 0.5 down	CGCGTTATGACAATTTTATGTAGATATCC
Tel VI R 1.75 fwd	GCGCAATACCCTGTAGTAGTCG
Tel VI R 1.75 rev	CGGCATGTAGACTTTACATATCTCG
Tel VI R 2.5 up	GCAATGAATCTTCGGTGCTTGG

Tel VI R 2.5 down	CCATACCAATATCAACTTCACGG
Tel VI R 5 fwd	CCCCGCCTTTGAAGATTGTCCC
Tel VI R 5 rev	CGAGACCCACTTGTATTCTTAGTGC
Tel VI R 15 up	GCGCAATATATAGCAGAAGAGC
Tel VI R 15 down	CAATTCGTCGATAAAGTGC
VPS15 5' fwd	GGAAGGCATACAGTATAATGGGGG
VPS15 5' rev	GCCCTTATACGTTGGAGAAAAGGT
VPS15 center fwd	CCATCACTGATTCGGAAGAATTAGTAGTG
VPS15 center rev	GATAGAGAATGCAGTACACTTCGGC
VPS15 3' fwd	GCCTAAATGAGCTTTCTTCTTCTAAAGCAG
VPS15 3' rev	GGAAGATTCCAATAAGCCCTGAGTTATC
YFR054C fwd	GGAAGATGCATTCGTCGATTGG
YFR054C rev	CCATCTGATTATATTGCATGCTCC

2.12 Chromatin immunoprecipitation and hybridization on tiling array (ChIP-chip)

2.12.1 ChIP-chip sample preparation

For ChIP-chip in *sir2*Δ (AEY2582) and *sas2*Δ *sir2*Δ strains (AEY5073), 200 OD of cells were harvested and ChIP was performed as described above (2.10.3). For each strain, subjected to ChIP-chip analysis, three independent biological replicates were used. From one chromatin preparation, at least 3 x 10 OD for α-H4 K16Ac ChIP, 3 x 10 OD for α-H4 ChIP, 1 x 10 OD for w/o antibody and 8 x 5 OD for input were prepared. The following antibodies were used: α-H4 K16Ac (Active Motif, #39167, Lot 01008001) and α-H4 (Millipore, #05-858, Lot DAM1794389). For subsequent processing, a minimum of 300 – 400 ng was required for IPs and up to 7000 ng of input DNA, which was used to determine the conditions for digestion with DNaseI (2.12.2). The amount of precipitated DNA was determined by measuring the DNA content of the combined samples using the NanoDrop spectrophotometer (Peqlab). One chromatin preparation of one replicate from one strain resulted in two tiling arrays (H4 K16Ac, H4), whereas input DNA was not hybridized to arrays.

2.12.2 Processing and hybridization of ChIP DNA

Tiling arrays representing the whole *S. cerevisiae* genome with ~3.2 million perfect match/mismatch probe pairs tiled at an average resolution of 5 bp (Affymetrix GeneChIP S. cerevisiae Tiling 1.0R arrays) were used. ChIP DNA (300 ng) was fragmented by limited DNaseI digestion to an average size of approximately 200 bp and labeled with Terminal Deoxynucleotidyl Transferase using the reagents of the Human Mapping 250K Sty Assay Kit (Affymetrix). Hybridization, washing and scanning of tiling arrays was performed according to the Affymetrix Chromatin Immunoprecipitation Assay protocol. Hybridization was carried out by Ludger Klein-Hitpass, Institut für Zellbiologie, Universitätsklinikum Essen.

2.12.3 Tiling array data analysis

The intensities measured for perfect match probes were extracted from the binary cel files along with their coordinates in the *S. cerevisiae* genome as annotated by Affymetrix. The intensities were transformed by taking the natural logarithm of the intensities (log-intensities). Data quality assessment, as well as data normalization and data analysis identifying regions enriched or depleted for H4 K16Ac was performed using the Bioconductor package "Starr" (Zacher et al. 2010). The mean values of the normalized log-intensities across the three replicates for H4 K16Ac were further normalized for the nucleosome density by subtracting the corresponding average values for histone H4 both from *sir2*Δ and *sas2*Δ *sir2*Δ samples. To identify whether depletion of H4 K16Ac in *sas2*Δ cells (Heise 2011) is due to an activity of Sir2, the mean log-intensities for H4 K16Ac in *sas2*Δ *sir2*Δ were normalized by subtracting the corresponding average values for H4 K16Ac in *sir2*Δ samples, which should reveal changes of the H4 K16Ac level upon deleting *SAS2* in *sir2*Δ cells. Bioinformatic analysis was performed by Ann Ehrenhofer-Murray, Arbeitsgruppe Molekulare Zellbiologie, Institut für Biologie, Humboldt-Universität zu Berlin, and by Ho-Ryun Chung, Department of Computational Molecular Biology, Max-Planck-Institut für molekulare Genetik, Berlin.

2.13 RNA extraction from *Saccharomyces cerevisiae,* cDNA synthesis and quantification (qRT-PCR)

For RNA extraction and subsequent cDNA synthesis, yeast cultures were grown to an OD of 0.9 to 1.5. Cells in 50 ml culture were harvested by centrifugation, frozen in liquid nitrogen and stored for about 16 hours at -80°C. 10 ml peqGOLD TriFast (Peqlab) was added to the pellet and vortexed with about 5 ml acid-washed glass beads for 5 min. After 5 min of incubation at room temperature, the mixture was transferred to peqGOLD PhaseTrap A tubes (Peqlab), 10 ml chloroform were added, it was mixed and the tubes were centrifuged at 1500 g until a clear phase layering was achieved. The upper phase was taken to precipitate the contained RNA by adding 5 ml isopropanol over night at -20°C. The next day, the precipitation mixture was centrifuged at 10000 g for 30 min, the pellet was washed with 5 ml of 70% ethanol, air-dried and dissolved in 100 µl of DEPC-treated water. 2.5 µg of extracted RNA was digested with TURBO DNase (Ambion, Life Technologies) according to the manufacturer's protocol. First strand cDNA synthesis was then performed with the SuperScript III First-Strand Synthesis system for RT-PCR (Invitrogen, Life Technologies) according to the manufacturer's protocol using oligo(dT) primers to synthesize mRNA-corresponding cDNAs. For quantification of cDNAs corresponding to *GAL10* mRNAs, qPCR was performed with the GAL10 5' fwd/rev primers (Table 6). *ACT1* expression was used as an internal control (amplified in qPCR with the ACT1 up (2) and ACT2 primers).

3 Results

3.1 Cell-cycle dependence of Sas2-mediated H4 K16 acetylation

The packaging of eukaryotic DNA into chromatin is not static, but rather faces many dynamic changes during the cell cycle in processes such as DNA replication, transcription and DNA repair. Among others, one fundamental step in these chromatin-challenging processes is the disassembly and reassembly of nucleosomes, which is specifically mediated by chromatin assembly factors. When DNA is replicated in S-phase, chromatin structure is disrupted ahead of the replication machinery and reestablished on the newly synthesized DNA strands. The histone acetyltransferase (HAT) Sas2, which catalyzes the acetylation of lysine 16 of histone H4 (H4 K16Ac) in *Saccharomyces cerevisiae*, has previously been shown to interact with the chromatin assembly factors Asf1 and CAF-1 (Meijsing and Ehrenhofer-Murray 2001), the latter of which is mainly active during S-phase (Smith and Stillman 1989; Kaufman et al. 1997). Both these chromatin assembly factors interact with the sliding clamp PCNA. These facts, as well as previous experiments (Heise 2011), led to the hypothesis that Sas2-mediated H4 K16 acetylation might be introduced on newly synthesized DNA in a replication-coupled manner.

To address the cell-cycle dependency of Sas2-mediated H4 K16 acetylation, we took advantage of a heat-inducible degron to rapidly switch on and off Sas2. For this purpose, *SAS2* was N-terminally fused to a temperature-sensitive fragment of mouse dihydrofolate reductase (DHFR) (Dohmen et al. 1994), which contains a cryptic N-degron that is only activated at the restrictive temperature of 37°C, leading to the degradation of the protein. Additionally, the degradation process is accelerated by simultaneous overexpression of the E3 ubiquitin ligase Ubr1, which is regulated by a galactose-inducible promoter (Labib et al. 2000; Makise et al. 2008). Moreover, this Sas2 heat-inducible degron fusion protein, termed Sas2-td, contained one N-terminal HA-tag, but unfortunately could not be detected by Western blotting. The strain construction and testing for complementation of Sas2-td was performed by Franziska Heise (Heise 2011).

3.1.1 Cell-cycle dependent acetylation of bulk histone H4 at K16 by Sas2

The histone chaperones Asf1 and CAF-1 cooperate to reassemble nucleosomes on newly synthesized DNA during S-phase. The HAT Sas2, which catalyzes H4 K16 acetylation, interacts with both these chromatin assembly factors. Therefore, the question arose whether there were differences in the acetylation of H4 K16 between cells that underwent DNA

replication in S-phase and cells that remained arrested in G1-phase and did not pass through S-phase.

In order to address this issue, we performed cell cycle experiments using cells that bear the repressible Sas2-td construct as described above (3.1.). Cells were arrested in G1-phase with the yeast mating pheromone α-factor in a Sas2 "off" state by growing them at the restrictive temperature (37°C) in medium containing galactose in order to degrade Sas2-td and to decrease H4 K16Ac levels. Subsequently, half of the cells were released from G1 arrest into S-phase, and Sas2-td activation and H4 K16 acetylation was induced by shifting the cells to 30°C and medium containing glucose (Figure 5A). The other half of the cells was maintained in G1 arrest and Sas2-td was activated as above. To further analyze the influence of the chromatin assembly factors CAF-1 and Asf1 on the appearance of H4 K16 acetylation, the described cell-cycle experiment was performed in cells carrying the Sas2-td construct in wild type, *cac1*Δ and *asf1*Δ genomic backgrounds. The construction of the strains bearing Sas2-td in *cac1*Δ and *asf1*Δ background was performed by Franziska Heise.

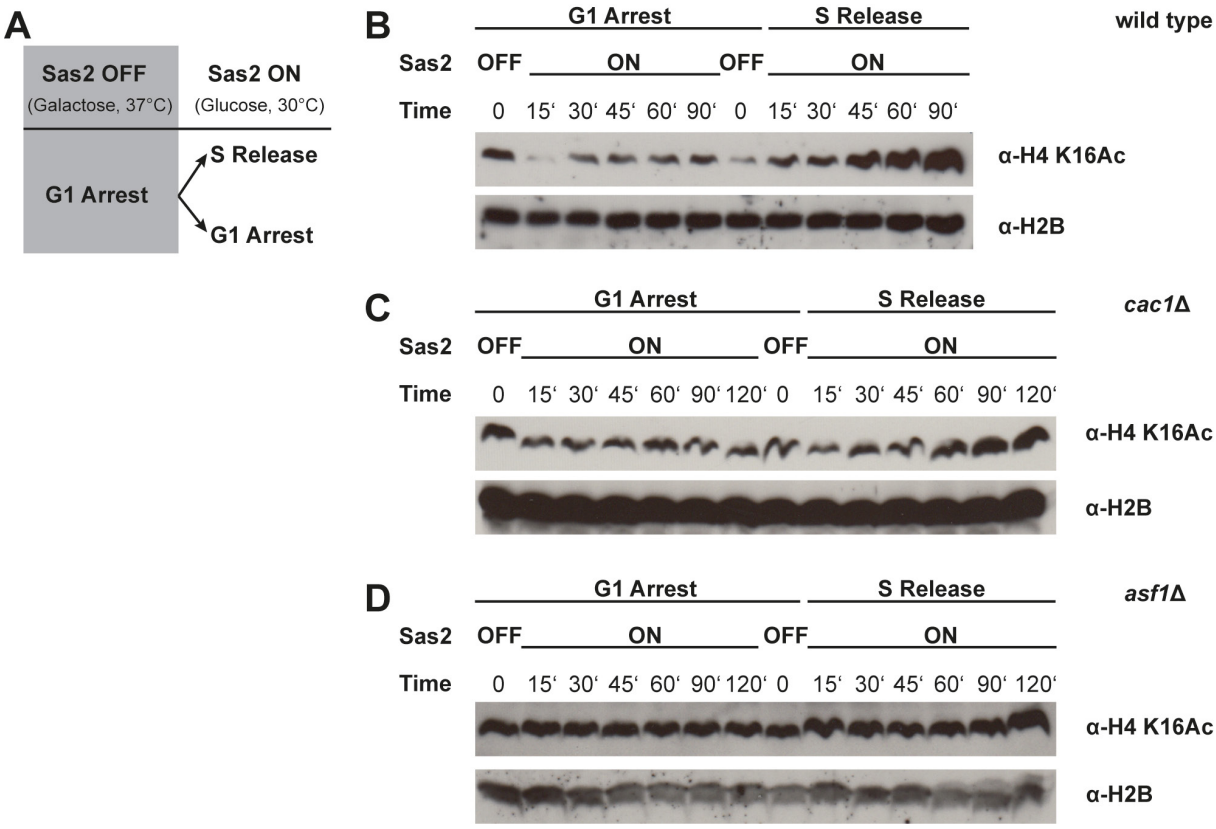

Figure 5. Sas2-mediated H4 K16 acetylation was dependent on progression through S-phase in wild type cells whereas H4 K16 acetylation was delayed or lacking upon deletion of *CAC1* or *ASF1*, respectively.
(**A**) An asynchronous culture with Sas2-td in wild type (AEY4488), *cac1*Δ (AEY4810) or *asf1*Δ (AEY4812) background, respectively, was grown over night at 37°C in medium containing galactose to shut *SAS2* off. The next day, cells were arrested in G1-phase by addition of α-factor, while keeping *SAS2* off. Subsequently, the culture was split: one half was released into S-phase by degradation of α-factor with pronase, the other half remained arrest in G1-phase by α-factor. In both cases *SAS2* was switched on by growing the cells at 30°C in

medium containing glucose. Samples for whole cell protein extracts were taken at time points indicated in B to D. Cell cycle phase was monitored by FACS analysis (not shown). (**B-D**) Western blot analysis of H4 K16Ac levels in G1-arrested and S-phase-released cells of wild type, *cac1*Δ or *asf1*Δ background, respectively. (**B**) G1-arrested wild type cells showed no increase in H4 K16Ac upon Sas2-td activation compared to S-phase-released cells, whereas similar levels for histone H2B were detected for both cell cycle stages. (**C**) In *cac1*Δ background, the increase of H4 K16Ac in S-phase was delayed and less pronounced compared to wild type. (**D**) In *asf1*Δ background, there were no differences in H4 K16Ac levels between G1-arrested and S-phase-released cells upon Sas2-td activation.

As expected, H4 K16 acetylation on bulk histones was reduced, but not absent in G1-arrested cells in the absence of Sas2 (Figure 5B, time point 0), which is in line with the fact that Sas2 is responsible for about 60% of cellular H4 K16Ac (Heise et al. 2012). The residual H4 K16Ac could be due to an incomplete degradation of Sas2-td or to H4 K16 acetylation activity of other HATs, for instance Esa1 (Smith et al. 1998). Significantly, upon activation of Sas2-td, H4 K16Ac levels increased in cells that were released into S-phase. Surprisingly, however, H4 K16Ac levels remained low in G1-arrested cells (Figure 5B). Notably, the observed cell-cycle dependent differences in H4 K16 acetylation were not a consequence of cell-cycle dependent differences in the activation of Sas2-td, since the same experimental procedure with cells carrying the Sas2-td construct with six additional, C-terminal HA-tags showed no difference in Sas2-6xHA levels between G1- and S-phase (Figure 6A). Thus, the increase in H4 K16Ac levels in cells in S-phase could be attributed to acetylation activity of Sas2 that is coupled to an event during S-phase, for instance replication-associated chromatin assembly.

Since Sas2 interacts with CAF-1, which assembles nucleosomes on newly replicated DNA, a deletion of its subunit *CAC1* might influence H4 K16Ac levels during S-phase. In *cac1*Δ background, Sas2-mediated H4 K16Ac levels also only increased in cells that were released into S-phase, whereas G1-arrested cells showed no increase in H4 K16Ac (Figure 5C). However, compared to cells with a wild type genomic background, the increase in H4 K16Ac levels in S-phase-released cells was delayed, which may be due to a slower nucleosome assembly rate in the absence of functional CAF-1 or to a lack of interaction with Sas2.

The histone chaperone Asf1, as CAF-1, is also involved in replication-coupled reassembly of nucleosomes and it also interacts with Sas2. Importantly, in the *asf1*Δ background, Sas2-mediated H4 K16Ac levels did not change upon activation of Sas2-td neither in cells arrested in G1-phase nor in cells released into S-phase, when compared with cells in which *SAS2* was "off" (Figure 5D). In both G1- and S-phase, Sas2-td was detected in equal amounts (Figure 6B), showing that a deletion of *ASF1* did not affect the expression of Sas2-td and only influenced Sas2-mediated H4 K16 acetylation. The observed lack in H4 K16 acetylation upon

activation of Sas2-td suggested an important role for Asf1 in mediating the acetylation of H4 K16 by Sas2 during S-phase.

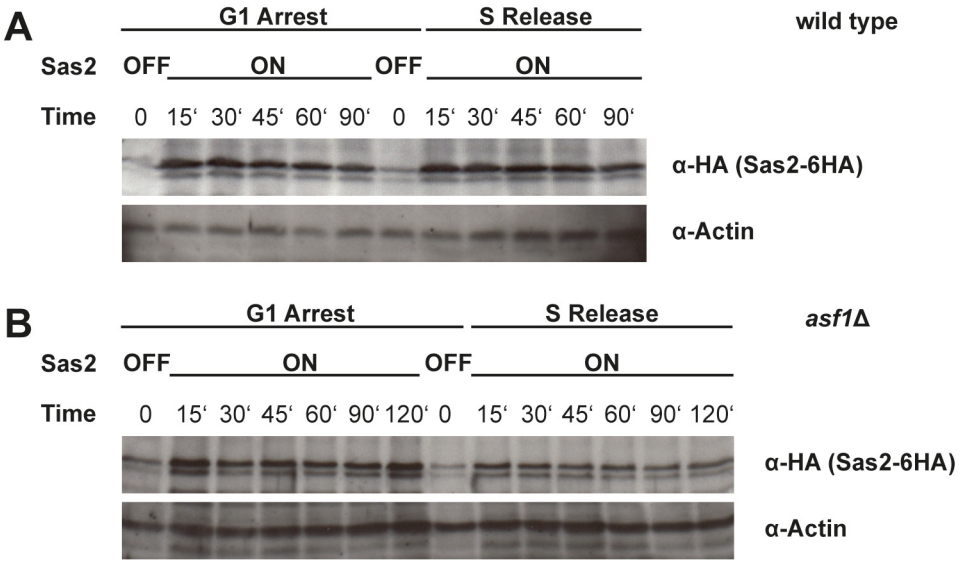

Figure 6. Sas2-td was equally activated in G1- and S-phase in cells carrying Sas2-td in wild type or *asf1*Δ genomic background.
Strains carrying a Sas2-td variant with six C-terminal HA-tags were constructed for wild type (AEY5495) and *asf1*Δ (AEY5499) and subjected to cell cycle experiments as depicted schematically in Figure 5A and described in the text. (**A**) Sas2-td was equally activated in G1- as well as in S-phase in wild type background. (**B**) Sas2-td was also equally activated in G1- and S-phase in *asf1*Δ background. Actin served as loading control.

Since the above experiments only gave information about acetylation of K16 of bulk histone H4 and not about its incorporation into nucleosomes, in a next step cell-cycle dependent incorporation of H4 K16Ac into the chromatin was investigated by chromatin immunoprecipitation experiments (ChIP-chip).

3.1.2 No difference in chromatin-bound H4 K16Ac between cells in S- and G1-phase

In the experiments above (3.1.1), Sas2-mediated H4 K16 acetylation was found to require passage through S-phase. This raised the question whether histone H4 acetylated at K16 is also incorporated into chromatin in a cell-cycle dependent manner. In order to address this question, we performed the same cell-cycle experiment described above (Figure 5A), using the yeast strain with the Sas2-td construct in wild type background. Samples of cells for chromatin immunoprecipitation (ChIP) were taken when the cells were arrested in G1-phase while *SAS2* was switched off (time point “0”), and one hour after activation of Sas2-td in cells that were released into the following S-phase and in cells that remained arrested in G1-phase, respectively (time points “S release” and “G1 arrest”). Chromatin immunoprecipitation was performed with antibodies against histone H4 acetylated at K16 and unmodified histone H4,

respectively. For each time point, the immunoprecipitated DNA as well as input DNA were hybridized to high-resolution tiling arrays (ChIP-chip). To normalize H4 K16Ac levels to nucleosome density, ChIP-chip was also performed with an antibody against unmodified histone H4. The tiling arrays used in this experiment comprised 25-mer oligonucleotides with a 5 bp resolution (Affymetrix), thus tiling the complete genome of *S. cerevisiae*. The cell cycle experiment as well as the chromatin immunoprecipitations were carried out by Franziska Heise. The hybridization of the ChIP DNA onto the tiling arrays was performed by Ludger Klein-Hitpass (BioChip Labor, Universitätsklinikum Essen). The bioinformatic data analysis was done by Ho-Ryun Chung (Department of Computational Molecular Biology, Max-Planck-Institut für molekulare Genetik, Berlin).

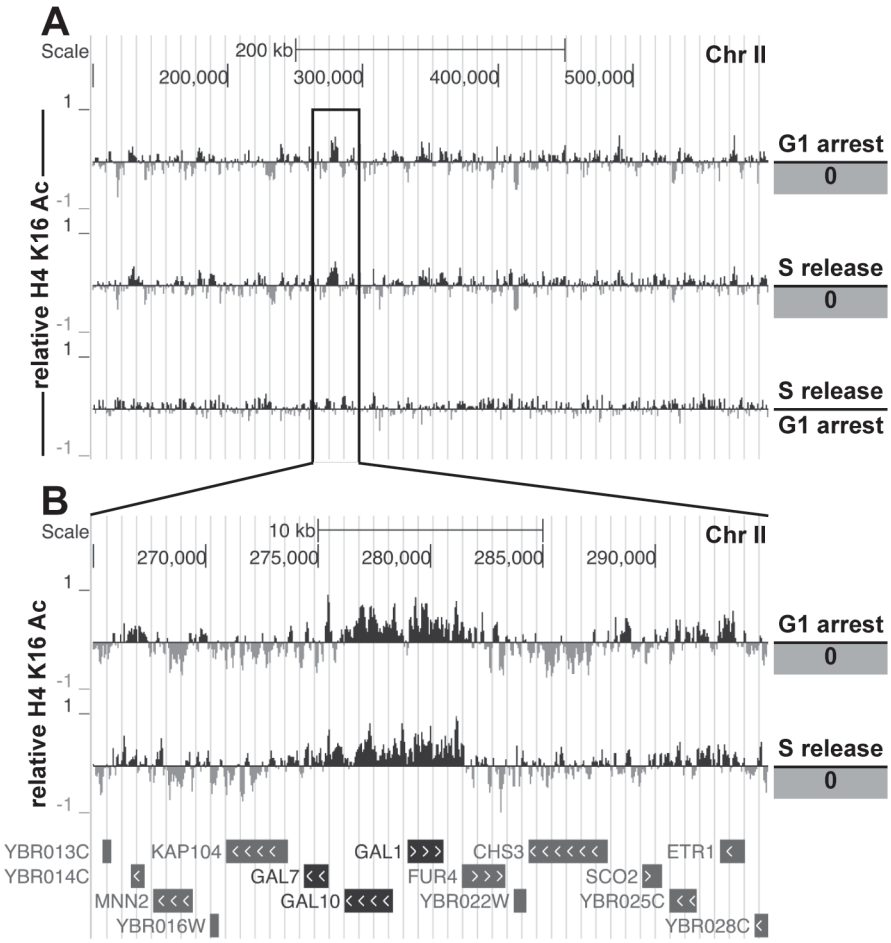

Figure 7. Chromatin incorporation of Sas2-dependent H4 K16Ac was similar in G1-arrested and S-released cells over the whole genome, but H4 K16Ac levels specifically increased at *GAL* genes in both cell cycle stages upon Sas2-td activation.
SAS2-td cells (AEY4488) were treated as above (Figure 5A), and samples were taken at time point "0" (before Sas2-td activation) and after one hour of Sas2-td activation either upon S-phase release ("S release"), or in cells maintained in G1-phase ("G1 arrest"). H4 K16Ac levels were determined relative to H4 levels by hybridization of ChIP samples to high-resolution tiling arrays (ChIP-chip). H4 K16Ac (relative to H4) was averaged over the whole genome for each analyzed sample and the change of relative H4 K16Ac comparing two samples (as indicated on the right) is shown. (**A**) H4 K16Ac levels (relative to H4) over position 100,000 to 600,000 of *S. cerevisiae* chromosome II. H4 K16Ac levels were increased at a few distinct loci in G1-arrested as well as in S-phase-released cells with activated Sas2-td compared to cells in which Sas2 was "off" ("0") (first two rows).

Comparing chromatin-bound H4 K16Ac levels between S-phase-released and G1-arrested cells (third row), the differences between both samples were insignificantly small. (**B**) Genomic locus of chromosome II marked with a frame in (**A**). H4 K16Ac levels in G1-arrested as well as S-phase-released cells increased at *GAL7*, *GAL10* and *GAL1* upon activation of Sas2-td compared to G1-arrested cells in which Sas2 was "off". Bioinformatic analysis was performed by H.-R. Chung. Data was visualized using the UCSC Genome Browser (http:// http://genome.ucsc.edu/).

Sas2-dependent H4 K16 acetylation was shown above to be coupled to the S-phase of the cell cycle. Nonetheless, the genome-wide analysis of chromatin-bound H4 K16Ac levels upon Sas2-td activation revealed only few sites of H4 K16Ac incorporation into chromatin. Comparing H4 K16Ac levels in G1-arrested or S-phase-released cells upon activation of Sas2-td with H4 K16Ac levels when Sas2-td was switched off, the levels of H4 K16Ac were similar in S- and G1-phase upon Sas2-td activation, i.e. the incorporation of H4 K16Ac was independent of the cell cycle stage (Figure 7A, Figure 8A). Moreover, H4 K16Ac levels increased only at a few genomic loci upon activation of Sas2-td in G1-arrested as well as S-phase-released cells. Interestingly, H4 K16Ac levels were increased at the galactose-inducible genes *GAL1*, *GAL7*, *GAL10* (Figure 7B) and to some extent at *GAL2* and *GAL3* (data not shown) in both S-phase-released and G1-arrested cells upon Sas2-td activation. Additionally, H4 K16Ac levels were also elevated at the heat shock gene *SSA1* in cells, in which Sas2-td was activated, compared to cells in which Sas2-td was "off" (Figure 8B). The observed cell cycle independent incorporation of H4 K16Ac at galactose-inducible *GAL* genes and the heat shock gene *SSA1* might be a consequence of transcriptional repression of these genes. To degrade Sas2-td in the *SAS2-td* strain, cells were grown in medium containing galactose at 37°C, thereby inducing the expression of galactose-inducible genes as well as heat shock genes. By shifting the cells to medium containing glucose and 30°C to activate Sas2-td, the galactose-inducible genes and the heat shock genes became transcriptionally repressed because of the changed culturing conditions. Thus, these ChIP-chip experiments suggested that H4 K16Ac might be incorporated into chromatin during transcriptional repression of a gene, which was in agreement with earlier studies (Kurdistani et al. 2004; Liu et al. 2005; Heise et al. 2012).

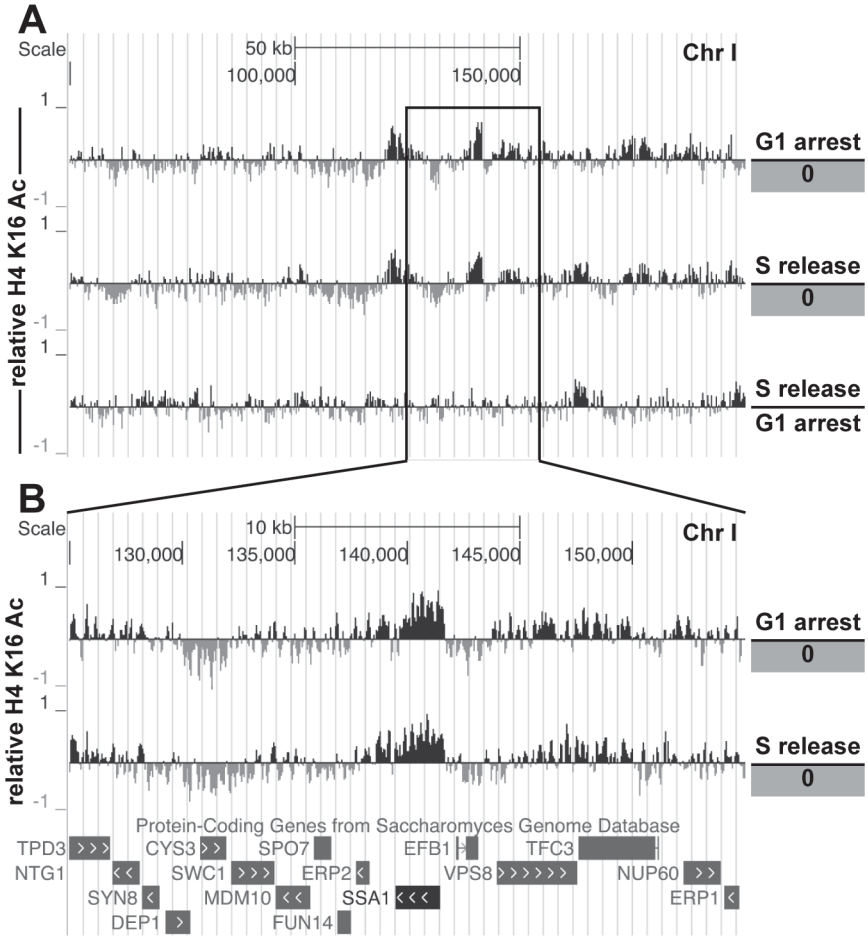

Figure 8. Chromatin incorporation of Sas2-dependent H4 K16Ac was similar in G1-arrested and S-released cells over the whole genome, but H4 K16Ac levels specifically increased at the heat shock gene *SSA1* gene in both cell cycle stages upon Sas2-td activation.
Samples were treated and ChIP-chip data was normalized as described above (Figure 7). (**A**) H4 K16Ac levels (relative to H4) over the *S. cerevisiae* chromosome I from position 50,000 to 200,000. H4 K16Ac levels were increased at a few distinct loci in G1-arrested as well as in S-phase-released cells with activated Sas2-td compared to cells in which Sas2 was "off" ("0") (first two rows). Comparing chromatin-bound H4 K16Ac levels between S-phase-released and G1-arrested cells (third row), the differences between both samples were small. (**B**) Genomic locus of chromosome I marked with a frame in (**A**). H4 K16Ac levels in G1-arrested as well as S-phase-released cells increased at the heat shock gene *SSA1* upon activation of Sas2-td compared to G1-arrested cells in which Sas2 was "off". Bioinformatic analysis was performed by H.-R. Chung.

The results of the ChIP-chip experiments showed that the chromatin incorporation of acetylated H4 K16 was similar in cells in S- and G1-phase upon activation of Sas2-td. Due to the fact that for each sample ("0", "G1 arrest" and "S release") the H4 K16Ac values for each position in the genome had been averaged over the whole genome, this could lead to a "masking" of incorporation of H4 K16Ac for the following reason. If acetylated H4 K16 was incorporated equally over the whole genome, the mean H4 K16Ac level would increase. However, this increase in the mean H4 K16Ac level would not be detectable in the ChIP-chip profiles, because the mean H4 K16Ac value was used to normalize the H4 K16Ac level at each position of the genome. Thus, the ChIP-chip profiles presented here cannot account for changes in the mean H4 K16Ac levels in the respective samples.

In order to address whether there were absolute differences in the incorporation of H4 K16Ac between cells in S- and G1-phase upon Sas2-td activation, a similar chromatin immunoprecipitation approach was conducted as described above (Figure 5A) using the *SAS2-td* strain. Samples for ChIP were taken from G1-arrested cells, in which Sas2-td was "off" (time point "0"), as well as from S-phase-released or G1-arrested cells at intervals between 15 and 55 min after Sas2-td activation. ChIP was performed using the same antibody against acetylated H4 K16 as for the ChIP-chip experiment. For data normalization, ChIP was also performed against unmodified histone H4, here with a different antibody than in the ChIP-chip experiment due to limits in the availability of the antibody. The immunoprecipitated DNA was quantified by quantitative real-time PCR (qPCR) at several genomic loci.

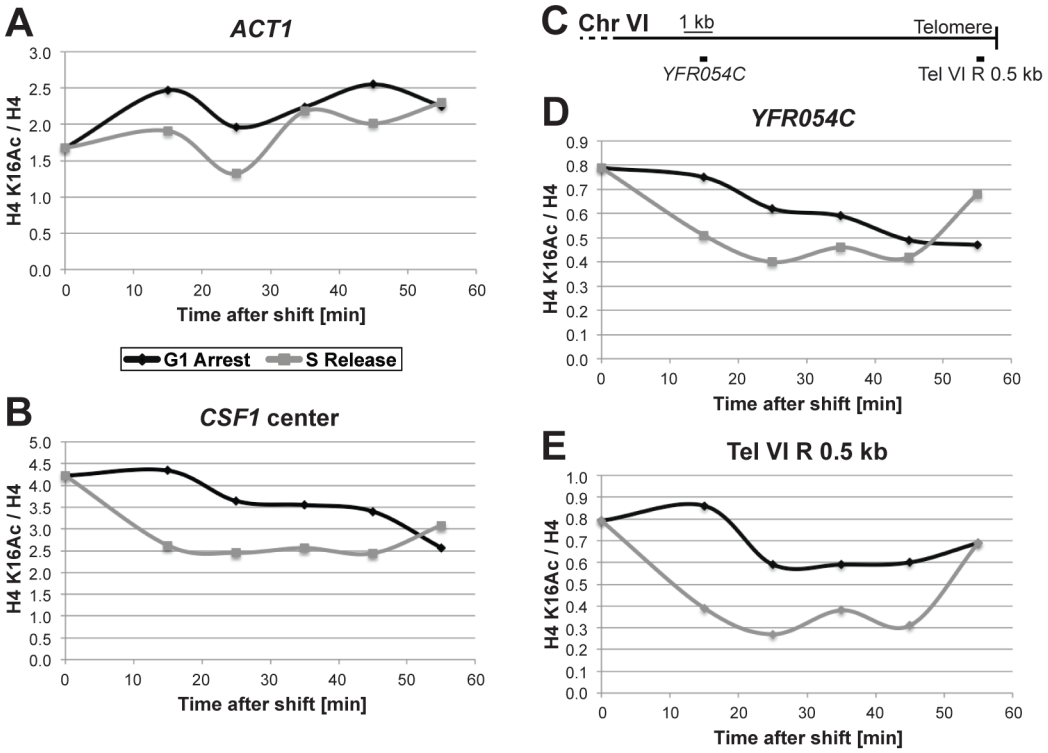

Figure 9. Upon Sas2-td activation, Sas2-mediated H4 K16Ac levels in G1-arrested stayed relatively constant, whereas H4 K16Ac levels in S-phase-released cells decreased and finally increased reaching higher H4 K16Ac levels than G1-arrested cells.
G1-arrested cells carrying the *SAS2-td* construct were released into S-phase or kept in G1-phase while activating Sas2-td. ChIP was performed against H4 K16Ac and unmodified H4 for normalization. The immunoprecipitated DNA was quantified by qPCR at the indicated loci. Cell cycle phase was monitored by FACS analysis (not shown). Results of one representative experimental replicate are shown. (**A, B**) Upon Sas2-td activation, H4 K16Ac levels in G1-arrested cells remained constant at the highly expressed *ACT1* gene (**A**) as well as at the poorly expressed *CSF1* gene (**B**), whereas H4 K16Ac levels in S-phase-released cells decreased and finally increased at both genes reaching a higher level of H4 K16Ac than in G1-arrested cells 55 min after S-phase release. (**C**) Schematic representation of the localization of the investigated loci at the right telomere of chromosome VI. (**D, E**) At both loci, the subtelomeric *YFR054C* gene (**D**) as well as at a telomere-proximal locus 0.5 kb away from the telomere of chromosome VI (**E**), upon Sas2-td activation, H4 K16Ac levels in S-phase-released cells decreased and finally increased after 55 min, whereas H4 K16Ac levels in G1-arrested cells remained constant (as seen for *ACT1* (**A**) as well as *CSF1* (**B**)).

Sas2-mediated H4 K16Ac on bulk histone H4 was shown above to be cell-cycle dependent. Bulk histone H4 is acetylated at K16 during S-phase in a Sas2-dependent manner. Thus, we expected that upon activation of Sas2-td, H4 K16Ac is incorporated into chromatin during S-phase, but not G1-phase. Unexpectedly, the chromatin-bound fraction of acetylated H4 K16 at the highly expressed *ACT1* gene (Figure 9A), the poorly expressed *CSF1* gene (Figure 9B) as well as two (sub)telomeric loci (Figure 9C-E) did not markedly differ between cells in G1- or S-phase upon Sas2-td activation. Thus, bulk histone H4, which was acetylated at K16 during S-phase, was not incorporated into chromatin during the same S-phase. Surprisingly, at all investigated loci, while H4 K16Ac levels in G1-arrested cells remained approximately constant, H4 K16Ac levels in S-phase-released cells decreased and subsequently increased 55 min after the release into S-phase, reaching a moderately higher level of H4 K16Ac than in G1-arrested cells at some, but not all loci (Figure 9). This decrease of H4 K16Ac in S-phase-released cells could be attributed to a dilution of H4 K16Ac due to genome and chromatin duplication. During S-phase, DNA and chromatin are duplicated. In the cell cycle experiment with the strain bearing the *SAS2-td* construct, Sas2-td was activated at the same time as the cells were released into S-phase. In these S-phase-released cells, Sas2 acetylated K16 on bulk histone H4, but this H4 K16Ac apparently was not incorporated into chromatin. This lack in the availability of H4 K16Ac to be incorporated into chromatin may explain the dilution of H4 K16Ac, which was observed during S-phase-coupled DNA and chromatin duplication. Possibly, the acetylated H4 K16 might be only incorporated into chromatin in the second S-phase after activation of Sas2-td, which was not analyzed with the described experiments. The increase of H4 K16Ac in S-phase-released cells 55 min after the release suggested a possible incorporation of H4 K16Ac later in the cell cycle. It remains to be investigated when acetylated H4 K16 is incorporated into chromatin and where and how it is stored in the intervening period between its catalysis and its chromatin incorporation. However, since the histone chaperones Asf1 and CAF-1 were involved in the Sas2-mediated acetylation of H4 K16, these chaperones might also mediate the storage of H4 K16Ac.

3.2 Sas2-mediated H4 K16Ac is deposited in genes upon repression

Chromatin immunoprecipitation experiments showed that when galactose-inducible genes as well as heat shock genes became repressed due to changes in culturing conditions, H4 K16Ac levels increased at these genes (Figure 7B, Figure 8B). The incorporation of acetylated H4 K16 itself does not cause gene repression. Instead, due to charge neutralization, histone acetylation is associated with an opening of the chromatin structure (Shogren-Knaak et al.

2006). Thus, the question arose how H4 K16Ac becomes enriched at repressed genes, which factors contribute to the enrichment of H4 K16Ac and which purpose this histone mark serves at repressed genes.

3.2.1 Sas2-mediated H4 K16Ac was incorporated during repression of *GAL* genes

As seen in the ChIP-chip experiments with the heat-inducible Sas2-degron construct (*SAS2-td*), H4 K16Ac became enriched at the galactose-inducible genes *GAL1*, *GAL7*, *GAL10* (Figure 7B) and to some extent at *GAL2* and *GAL3* (data not shown) upon repression of these genes due to a shift of the carbon source from galactose to glucose. We asked whether the observed deposition of H4 K16Ac in genes upon repression was also seen in wild type cells, without the complication of switching Sas2 on and off using Sas2-td, and whether this deposition of H4 K16Ac was also seen outside of S-phase. We decided to use the *GAL* genes as a model to study the enrichment of Sas2-mediated H4 K16Ac during their transcriptional repression.

In a first step, we tested whether wild type cells showed an enrichment of H4 K16Ac at *GAL* genes when these genes became repressed outside of S-phase. Initially, we also sought to address the increase of H4 K16Ac during the repression of the heat shock gene *SSA1*, which unfortunately was not observed in the course of the following experiments (data not shown). In order to investigate the enrichment of H4 K16Ac during the repression of the *GAL* genes, wild type cells, and as a control, cells with a deletion of *SAS2*, were shifted from galactose to glucose and from 37°C to 30°C. In order to eliminate effects of replication-coupled incorporation of acetylated H4 K16 into chromatin, the cells were arrested in G1-phase of the cell cycle by the α-factor mating pheromone. Samples of cells were taken for ChIP in medium containing galactose at 37°C (time point "0") and at regular intervals up to 60 min after the shift to glucose and 30°C. ChIP was performed using antibodies against histone H4 acetylated at K16 and for normalization against unmodified histone H4. H4 K16Ac levels were measured by qPCR at the *GAL10* and *GAL3* ORFs and normalized to H4 K16Ac levels at the control gene *ACT1* at the respective time points.

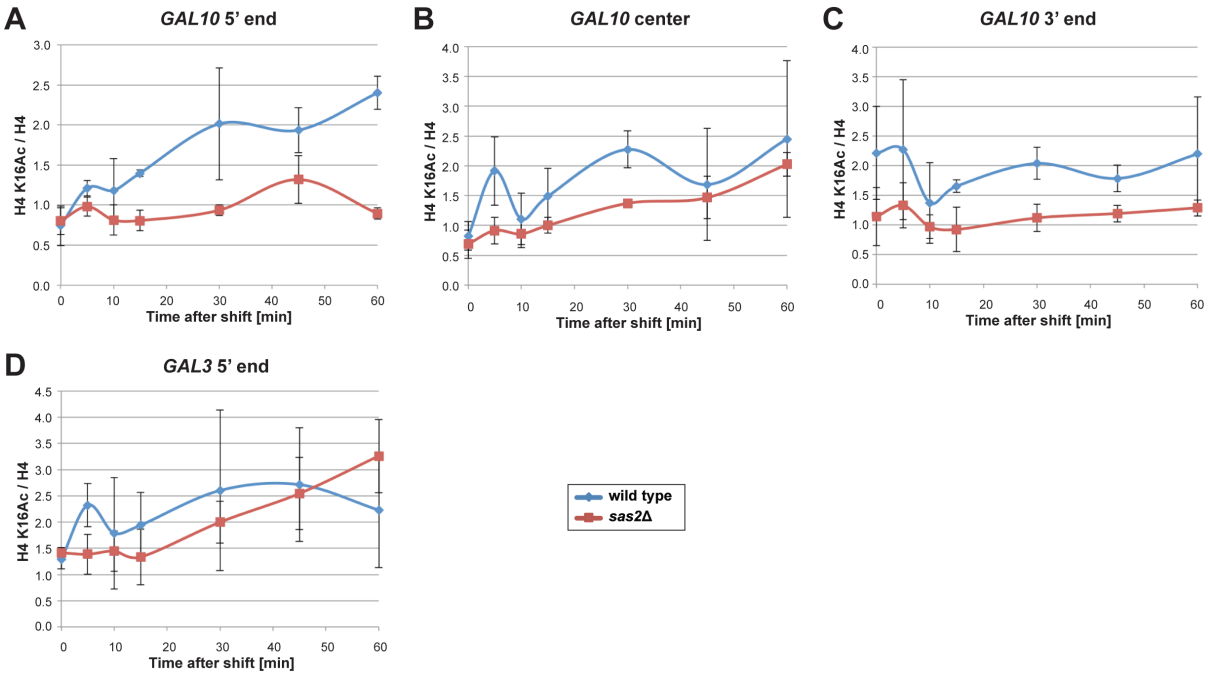

Figure 10. H4 K16Ac became enriched at *GAL10* upon its repression in wild type, but not in *sas2*Δ cells. Wild type and *sas2*Δ cells were shifted from medium containing galactose and 37°C to medium containing glucose and 30°C, keeping the cells arrested in G1-phase with α-factor. Samples of cells for ChIP were taken at the indicated time points (time point "0": in medium containing galactose at 37°C, following time points: in medium containing glucose at 30°C). G1-arrest was monitored by FACS analysis (not shown). ChIP was performed for H4 K16Ac and unmodified H4. Immunoprecipitated DNA was quantified by qPCR at the indicated loci. H4 K16Ac/H4-values were normalized to H4 K16Ac/H4-values at *ACT1* at the respective time points. (**A**) H4 K16Ac became enriched at the 5' end of the *GAL10* ORF in wild type cells and not in *sas2*Δ cells. This increase in acetylated H4 K16Ac was not that strong at the center of *GAL10* (**B**), the 3' end of the *GAL10* ORF (**C**), as well as the 5' end of the *GAL3* ORF (**D**). Error bars represent SD of three biological replicates.

Importantly, H4 K16Ac became enriched at the 5' end of the ORF of *GAL10* in wild type cells upon repression of the *GAL10* gene (Figure 10A). This enrichment was Sas2-dependent, because in *sas2*Δ cells, no such increase in H4 K16Ac levels could be seen. Moreover, the observed accumulation of H4 K16Ac was not a consequence of replication-coupled incorporation, because the cells were efficiently kept in G1-arrest over the whole experimental time course. At the center of *GAL10* (Figure 10B), as well as at the 3' end of the ORF of *GAL10* (Figure 10C) this increase of acetylated H4 K16 was not as pronounced as at the 5' end.

In order to investigate a galactose-inducible gene at a chromosome other than chromosome II, where *GAL10* is located, we chose *GAL3*, which also showed an increase of H4 K16 acetylation in the ChIP-chip experiments using the Sas2-td strain (3.1.2) and which is located on chromosome IV. Here, an increase in H4 K16Ac after the shift from galactose to glucose could be seen in wild type cells, but surprisingly the increase was similar in *sas2*Δ cells (Figure 10D). In contrast to *GAL10*, *GAL3* is not a metabolic gene, but instead is a

transcriptional regulator for the expression of the *GAL* metabolic genes (*GAL1*, *GAL10*, *GAL2* and *GAL7*). Therefore, the basal transcription rate of *GAL3* in glucose might be higher than that of *GAL10*, leading to a less pronounced enrichment of H4 K16Ac at *GAL3* upon the shift from galactose to glucose. Additionally, in *sas2*Δ cells, H4 K16Ac levels at *GAL3* increased starting at 30 min after the shift to glucose. This increase likely is due to the catalytic activity of another HAT, for instance Esa1, but this was not further investigated here.

Although all experiments presented here and in the following sections were carefully repeated to obtain three biological replicates, in some cases standard deviations were high. These biological variations could not be completely excluded, sometimes leading to results lacking statistical significance, which nevertheless were considered to constitute biological effects, e.g. in the comparison of effects in different strains. Reasons for these biological variations will be discussed below (4.5.1).

The HAT Sas2 has been shown to interact with the chromatin assembly factors CAF-1 and Asf1 (Meijsing and Ehrenhofer-Murray 2001), which are involved in the assembly of nucleosomes on replicated DNA. In contrast to CAF-1, whose chromatin assembling activity is almost completely limited to replicated DNA (Smith and Stillman 1989; Kaufman et al. 1997), Asf1 is also involved in chromatin dynamics during transcription, where it disassembles nucleosomes ahead of the RNA polymerase II, and it is also involved in the reassembly of nucleosomes when the transcription machinery has passed through (Schwabish and Struhl 2006). Next to CAF-1 and Asf1, there are other chromatin assembly factors that are known to be involved in nucleosome dis- and reassembly during transcription. Among these are the HIR chromatin assembly complex, Rtt106 and Spt6 (Imbeault et al. 2008; Avvakumov et al. 2011).

Because of the known interactions of Sas2 with the chromatin assembly factors CAF-1 and Asf1, in a next set of experiments, we addressed whether these factors influence H4 K16Ac incorporation during transcriptional repression of the *GAL10* gene. Additionally, the other known chromatin assembly factors participating in transcription-coupled nucleosome dis- and reassembly, HIR, Rtt106 and Spt6, were investigated regarding their influence on the enrichment of acetylated H4 K16 during repression of *GAL10*. In order to address this, cells with a deletion of *CAC1* (the largest subunit of CAF-1), *ASF1*, *HIR1* (a subunit of the HIR complex) or *RTT106*, respectively, were shifted from medium containing galactose at 37°C to glucose at 30°C while keeping the cells arrested in G1-phase with α-factor. A deletion of the *SPT6* gene is lethal. Therefore, a temperature-sensitive *SPT6* mutant (*spt6-1004*) was used and treated as follows: After having grown the cells at 37°C in medium containing galactose

for two hours, cells were shifted to glucose but maintained at 37°C. The temperature of 37°C efficiently inactivates Spt6-1004. Simultaneously, the cells were also maintained in G1-arrest. For all investigated mutant strains, samples of cells were taken for ChIP in galactose (time point "0") and at regular intervals up to 60 min after the shift to glucose. ChIP was performed against H4 K16Ac and, for normalization, against unmodified histone H4. H4 K16Ac levels were measured by qPCR at the 5' end of the *GAL10* ORF and compared to the obtained results for wild type and *sas2*Δ cells (Figure 11).

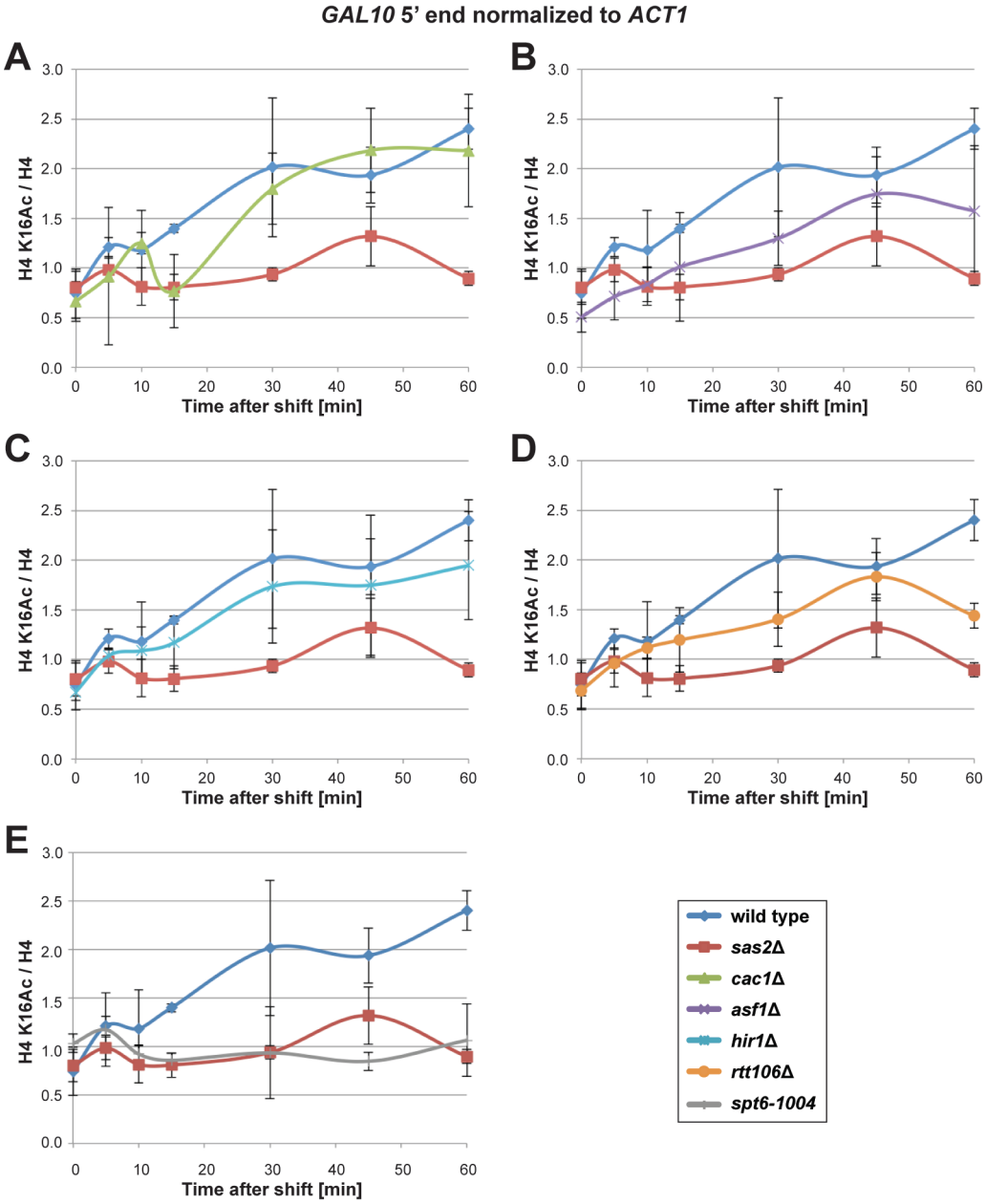

Figure 11. Effects of chromatin assembly factors on the deposition of H4 K16Ac at *GAL10* during transcriptional repression.
G1-arrested cells with deletions of *CAC1* (AEY5262, **A**), *ASF1* (AEY5281, **B**), *HIR1* (AEY5370, **C**), *RTT106* (AEY5380, **D**) and with the temperature-sensitive *spt6-1004* allele (AEY5412, **E**) were shifted from galactose- to glucose-containing medium. G1-arrest was monitored by FACS analysis (not shown). H4 K16Ac levels at the 5' end of the *GAL10* ORF were analyzed by ChIP. H4 K16Ac/H4-values were normalized to H4 K16Ac/H4-

values at *ACT1* at the respective time points. (**A, B, C**) H4 K16Ac in *cac1*Δ, *asf1*Δ and *hir1*Δ cells was enriched at *GAL10* upon its repression. (**D**) Deletion of *RTT106* caused an intermediate enrichment of H4 K16Ac at *GAL10*. (**E**) Disruption of Spt6 function in the *spt6-1004* strain led to an absence of H4 K16Ac enrichment. Error bars represent SD of three biological replicates.

As observed above, H4 K16Ac was enriched at the 5' end of the *GAL10* ORF upon *GAL10* repression in wild type cells (Figure 10A). Similar levels of H4 K16Ac enrichment were seen in strains with deletions of *CAC1* (Figure 11A) and *HIR1* (Figure 11C), which suggested that CAF-1 as well as HIR are not involved in repression-coupled incorporation of H4 K16Ac. There was a trend towards a slightly reduced incorporation of H4 K16Ac in *asf1*Δ cells (Figure 11B), but this was statistically not significant and therefore was not investigated in more detail. A deletion of *RTT106* reduced the enrichment of acetylated H4 K16 at *GAL10* (Figure 11D). This reduction was well pronounced 60 min after the shift to glucose, but less so at earlier time points. Thus, we concluded that Asf1 as well as Rtt106 to some extent influence the incorporation of H4 K16Ac upon gene repression.

The strongest effect in repression-coupled enrichment of H4 K16Ac at *GAL10* was seen for a disruption of Spt6 function in the *spt6-1004* mutant strain (Figure 11E). In these *spt6-1004* mutant cells, H4 K16Ac levels did not increase at *GAL10* after the shift from galactose to glucose. It is important to point out that the effects of the functional disruption of the investigated chromatin assembly factors on the enrichment of H4 K16Ac during transcriptional repression are not interfered by replication-coupled effects, because the cell samples used here were arrested in G1-phase.

The results so far indicated an involvement of Spt6 in the incorporation of acetylated H4 K16 into chromatin at genes that become transcriptionally repressed. Additionally, also Rtt106 seemed to influence the repression-coupled incorporation of H4 K16Ac. In the following two sections, the influences of Spt6 and Rtt106 on H4 K16Ac incorporation during transcriptional repression are investigated in more detail.

3.2.2 Influences of the chromatin assembly factor Spt6 on H4 K16Ac during gene repression

The essential nucleosome remodeling protein Spt6 has been shown to play a critical role in maintaining normal chromatin structure during transcription elongation, thereby repressing transcription initiation from cryptic promoters within open reading frames (Bortvin and Winston 1996; Hartzog et al. 1998; Kaplan et al. 2003). This suggests an important role for Spt6 in the redeposition of histones during transcriptional elongation (Schwabish and Struhl 2004). Furthermore, Spt6 is required for Set2-mediated trimethylation of H3 K36 (Youdell et

al. 2008), a histone mark that is associated with the 3' ends of coding regions, where it recruits the histone deacetylase Rpd3S. Rpd3S-mediated deacetylation keeps open reading frames in a hypoacetylated state, inhibiting transcription from cryptic promoters (Carrozza et al. 2005; Joshi and Struhl 2005; Li et al. 2007b). Furthermore, in *Schizosaccharomyces pombe*, Spt6 is a master regulator of nucleosome positioning and prevents transcription-coupled loss of histone marks (DeGennaro et al. 2013; Kato et al. 2013). Collectively, Spt6 plays an important role in the maintenance of chromatin structure during transcriptional elongation.

Initially, we sought to address which histone chaperones influence the incorporation of H4 K16Ac into chromatin during transcriptional repression. We observed above that in G1-arrested cells, a temperature-sensitive allele of *SPT6* (*spt6-1004)* caused a lack of enrichment of acetylated H4 K16 at the *GAL10* gene upon transcriptional repression, as was seen in *sas2*Δ cells (Figure 11D). This finding suggested an important role for Spt6 in the incorporation of H4 K16Ac into chromatin upon gene repression, which was investigated here in more detail. In order to address whether this effect was dependent on cell-cycle arrest in G1-phase, in a first approach, asynchronous cultures of wild type, *sas2*Δ and *spt6-1004* cells were shifted from galactose- to glucose-containing medium to repress *GAL10*. H4 K16Ac levels at *GAL10* were measured by ChIP.

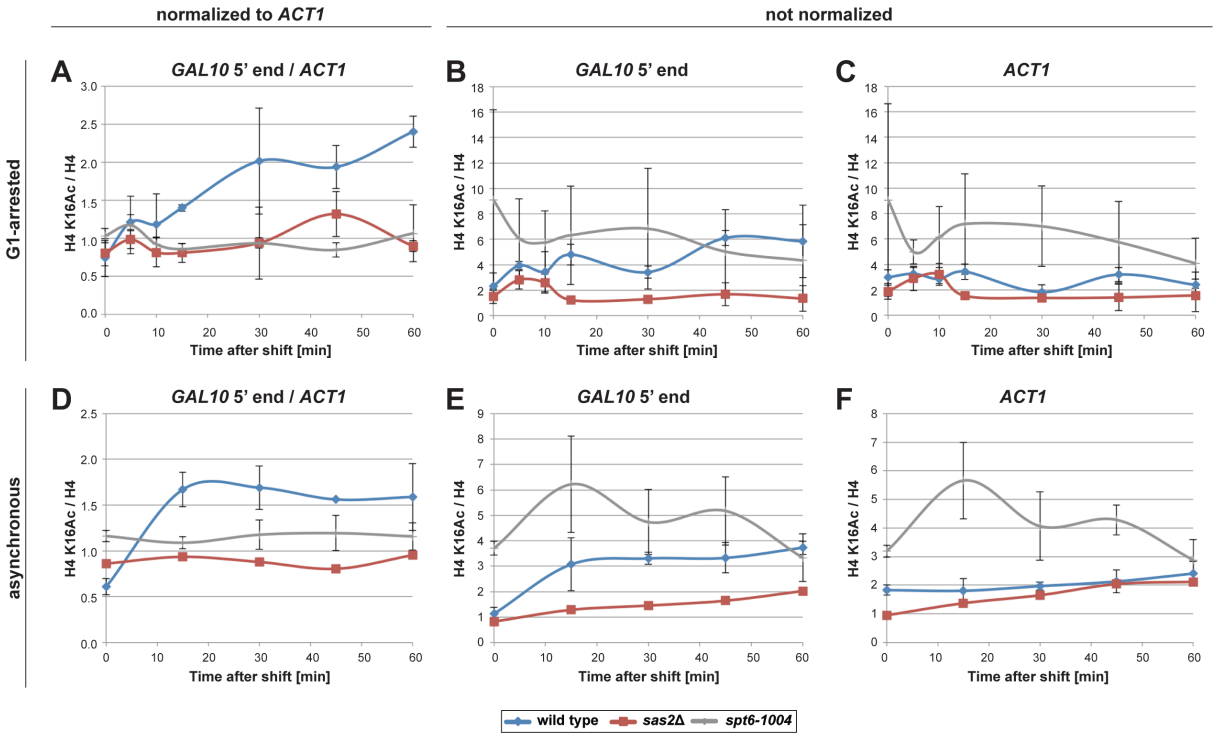

Figure 12. H4 K16Ac is not enriched in *spt6-1004* cells upon repression of *GAL10* due to already high levels of H4 K16Ac at *GAL10* as well as *ACT1*.

G1-arrested (**A, B, C**, results taken from the experiment in Figure 11E) and asynchronous cultures (**D, E, F**) of wild type (AEY5258), *sas2*Δ (AEY5260) and *spt6-1004* (AEY5412) cells were shifted from galactose- to glucose-containing medium to repress *GAL10* (time point "0" in galactose, following time points in glucose). H4 K16Ac levels were measured by ChIP at *GAL10*, normalized to *ACT1* H4 K16Ac levels (**A, D**). Non-normalized H4 K16Ac levels are shown for *GAL10* (**B, E**) and *ACT1* (**C, F**). (**A**) H4 K16Ac levels did not change in G1-arrested *spt6-1004* cells comparable to *sas2*Δ cells upon normalization of H4 K16Ac values at *GAL10* to *ACT1* (copy of Figure 11E for reasons of comparison). (**B**) When H4 K16Ac levels at *GAL10* were not normalized to H4 K16Ac at *ACT1*, H4 K16Ac in G1-arrested *spt6-1004* cells stayed at a level higher than in wild type cells, with no change upon shift from galactose to glucose. (**C**) At *ACT1* H4 K16Ac levels in G1-arrested *spt6-1004* cells were also strikingly higher than in wild type cells. (**D**) H4 K16Ac levels did not change in asynchronous *spt6-1004* cells comparable to *sas2*Δ cells upon normalization of H4 K16Ac values at *GAL10* to *ACT1*. (**E**) When H4 K16Ac levels at *GAL10* were not normalized to H4 K16Ac at *ACT1*, H4 K16Ac in asynchronous *spt6-1004* cells stayed at a level higher than in wild type cells, with no change upon shift from galactose to glucose. (**F**) At *ACT1* H4 K16Ac levels in asynchronous *spt6-1004* cells were also higher than in wild type cells. Error bars represent SD of three biological replicates.

As seen before and as in *sas2*Δ cells, H4 K16Ac levels in G1-arrested *spt6-1004* cells did not increase during transcriptional repression of *GAL10* (Figure 12A). A similar lack of H4 K16Ac enrichment for *spt6-1004* cells at *GAL10* was seen in asynchronous cultures, as in s*as2*Δ cells (Figure 12D). Interestingly, this low level of H4 K16Ac in *spt6-1004* cells was a result of the normalization of H4 K16Ac levels at *GAL10* to H4 K16Ac levels at *ACT1*. At both genes, *GAL10* and *ACT1*, non-normalized H4 K16Ac levels were higher than in wild type in G1-arrested (Figure 12B, C) as well as asynchronous cultures (Figure 12E, F). Thus, when H4 K16Ac values at *GAL10* were normalized to H4 K16Ac values at *ACT1*, the resulting normalized H4 K16Ac levels at *GAL10* in *spt6-1004* cells were low, comparable to *sas2*Δ cells in both G1-arrested (Figure 12A) as well as in asynchronous cultures (Figure 12D). However, H4 K16Ac levels at *GAL10* in *spt6-1004* cells did not increase as in wild type cells during the shift from galactose to glucose. Unexpectedly, H4 K16Ac levels in *spt6-1004* cells were also significantly higher at *ACT1* than in wild type cells (Figure 12C, F). These results so far suggested that, independently of the cell-cycle stage, in *spt6-1004* mutant cells, H4 K16Ac levels at the investigated genes did not depend on the expression level. Thus, Spt6 does not directly influence H4 K16Ac levels, but instead, this influence might be indirect by mediating the deposition of "K16 unacetylated" H4 during transcription. Because all H4 K16Ac values presented here are normalized to histone H4 occupancy, relative H4 K16Ac levels in *spt6-1004* cells were higher than in wild type cells.

In order to test whether *spt6-1004* mutant cells also showed high levels of H4 K16 acetylation at other genes whose expression is not regulated by galactose, ChIP experiments were performed to measured H4 K16Ac levels normalized to levels of histone H4 at highly expressed and poorly expressed genes in asynchronous cultures of wild type, *sas2*Δ and *spt6-1004* cells.

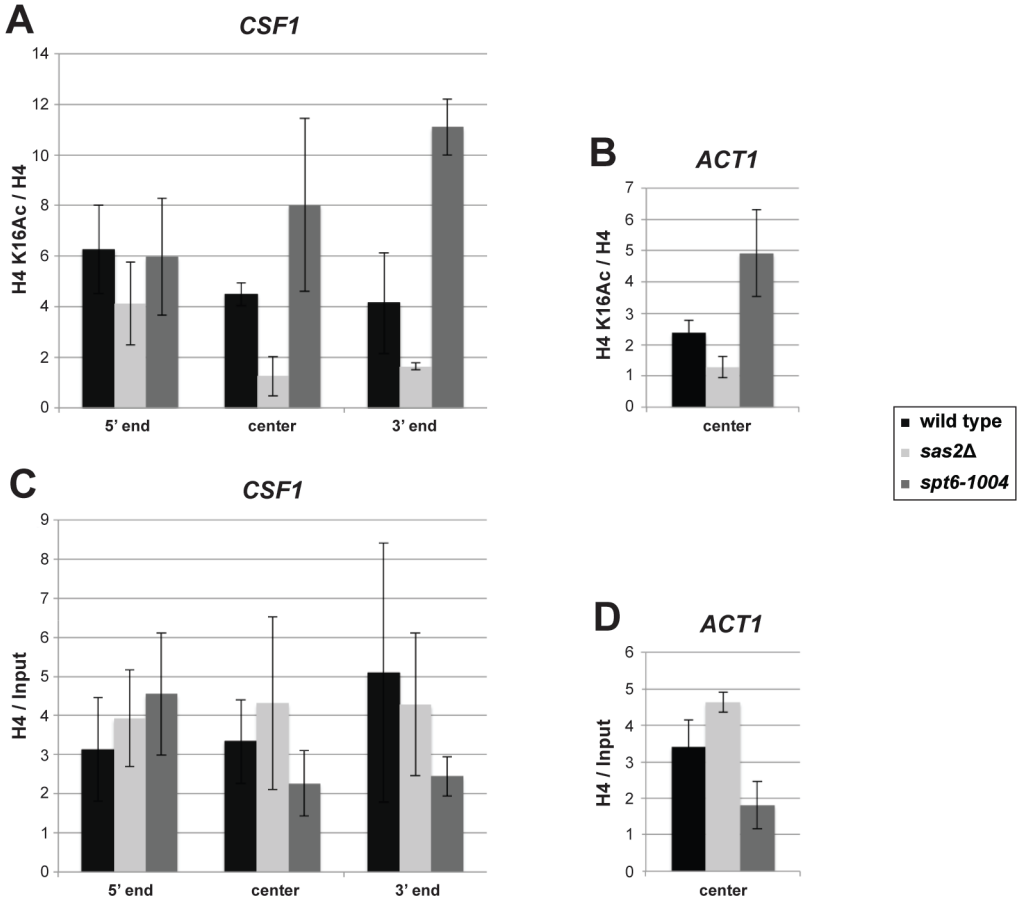

Figure 13. Relative H4 K16Ac levels at poorly expressed *CSF1* and at highly expressed *ACT1* were higher in *spt6-1004* than in wild type cells, whereas levels of histone H4 at *CSF1* and *ACT1* were lower in *spt6-1004* cells than in wild type cells.
H4 K16Ac levels were measured and normalized to H4 levels by ChIP at the poorly expressed *CSF1* gene and at the highly expressed *ACT1* gene in asynchronous cultures of wild type (AEY5258), *sas2*Δ (AEY5260) and *spt6-1004* (AEY5412) cells. (**A**) H4 K16Ac levels at *CSF1* were higher in *spt6-1004* than in wild type cells, especially at the center and the 3' end of the gene. (**B**) H4 K16Ac levels at *ACT1* were also higher in *spt6-1004* than in wild type cells. (**C**) In *spt6-1004* cell, less histone H4 was bound at the center and the 3' end of *CSF1* compared to wild type. (**D**) Also at *ACT1* H4 levels are lower in *spt6-1004* than in wild type cells. Error bars represent SD of three biological replicates.

Strikingly, in *spt6-1004* mutant cells H4 K16Ac levels at the center and the 3' end of the poorly expressed *CSF1* gene were higher than in wild type cells (Figure 13A). This effect could also be seen at the poorly transcribed gene *VPS15* (data not shown). Also, as seen before (Figure 12C, F), H4 K16Ac levels at the highly expressed *ACT1* gene were higher in *spt6-1004* than in wild type cells (Figure 13B). Previous work has shown that the histone occupancy at ORFs is reduced in *spt6*-1004 cells (Ivanovska et al. 2011). Consistent with this, the levels of histone H4 were decreased at the center and the 3' end of *CSF1* (Figure 13C) as well as at *ACT1* (Figure 13D) in *spt6-1004* compared to wild type cells. This reduction of histone H4 at poorly as well as highly expressed genes in *spt6-1004* cells might be a direct consequence of the loss of Spt6 function in the regulation of nucleosome occupancy during transcription elongation.

The results presented here so far suggested that Spt6 is not involved in the incorporation of H4 K16Ac into chromatin, because in *spt6-1004* cells relative H4 K16Ac levels at genes were even higher than in wild type cells. Instead, Spt6 might be involved in the eviction of acetylated H4 K16 during transcription and/or the incorporation of "K16 unacteylated" H4 in the wake of the transcription machinery and thereby indirectly influencing relative H4 K16Ac levels.

As others (Ivanovska et al. 2011) and we have seen, the histone occupancy at ORFs in *spt6-1004* cells is reduced compared to wild type cells. Moreover, Spt6 was shown to regulate transcription by controlling the chromatin structure over regulatory regions at gene promoters (Ivanovska et al. 2011). Considering that H4 K16Ac levels in *spt6-1004* cells at *GAL10* did not change upon shift from galactose to glucose, which represses *GAL10* expression in wild type cells, we asked whether *GAL10* was similarly repressed in *spt6-1004* cells upon this shift of medium. In order to address this, *GAL10* expression was measured by quantifying *GAL10* mRNAs transcribed into cDNAs (qRT-PCR). The constitutively expressed housekeeping gene *ACT1* was taken as internal control.

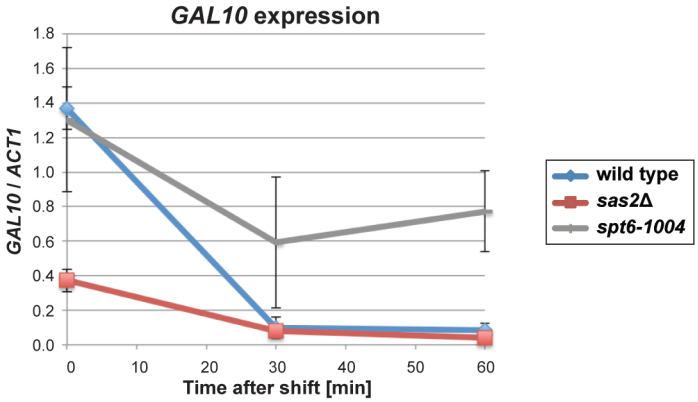

Figure 14. Upon shift from galactose to glucose, *GAL10* was not repressed in *spt6-1004* cells.
Total RNA was extracted from wild type (AEY5258), *sas2*Δ (AEY5260) and *spt6-1004* (AEY5412) cells upon shift from medium containing galactose (time point "0") to medium containing glucose (following time points). Amounts of *GAL10* transcripts were measured by qRT-PCR. For normalization, amounts of transcripts of the constitutively expressed *ACT1* gene served as internal control. In wild type cells, *GAL10* was completely repressed after the shift from galactose to glucose. In *sas2*Δ cells, *GAL10* expression in galactose-containing medium was much lower than in wild type cells (time point "0"), but *GAL10* was also completely repressed after the shift from galactose to glucose. In *spt6-1004* cells, levels of *GAL10* expression in galactose-containing medium was comparable to wild type (time point "0"), but *GAL10* expression was only reduced to about 50%, and not repressed, upon shift to glucose. Error bars represent SD of three biological replicates.

As expected for wild type cells, *GAL10* became strongly repressed upon shifting the cells from galactose to glucose medium (Figure 14). Astonishingly, *GAL10* expression in *sas2*Δ cells under inducing conditions was considerably lower than in wild type cells (Figure 14, time point "0"). Nevertheless, in glucose medium, *GAL10* also became completely repressed

in *sas2*Δ cells. *spt6-1004* cells in galactose had similar levels of *GAL10* expression as wild type cells (Figure 14, time point "0"). Interestingly, in *spt6-1004* cells under repressing conditions, *GAL10* was still expressed at a level of about 50% of the expression in galactose. One possibility is that this remaining expression of *GAL10* in *spt6-1004* cells in glucose medium could be due to the reduced histone occupancy at genes that became repressed resulting from the loss of Spt6 function, as was seen by Ivanovska et al. (2011). This reduced histone occupancy at *GAL10* in turn could result in a higher accessibility of the DNA for RNA polymerase II causing a remaining level of expression of *GAL10*.

Still, the question remained how Spt6 influences Sas2-mediated H4 K16 acetylation. The results presented here suggested that Spt6 is not involved in the incorporation, but rather in the eviction of H4 K16Ac during transcription, since relative H4 K16Ac levels at all investigated genes were not decreased in *spt6-1004* compared to wild type cells, instead they were even increased. If Spt6 is not involved in the incorporation of H4 K16Ac at genes that become repressed, then Spt6 is not expected to be present at repressed genes. Furthermore, Spt6 was characterized as nucleosome assembly factor (Bortvin and Winston 1996), and not as a factor mediating nucleosome disassembly. Thus, if Spt6 is involved in the deposition of K16-unacetylated H4 during transcription, then Spt6 is expected to be associated with actively transcribed genes. Previous work has shown that high expression levels of a gene correlate with Spt6 association with this gene (Ivanovska et al. 2011). In a first experiment, we therefore tested whether this was also the case for the genes investigated in this study, the poorly expressed *CSF1* and the highly expressed *ACT1*. In order to address this, ChIP was performed in cells bearing a 9myc-tagged version of Spt6 in wild type as well as in *sas2*Δ background. ChIP for Spt6 in *sas2*Δ background was performed to test whether Sas2-catalyzed H4 K16Ac might influence the chromatin association of Spt6. As a control, ChIP against the myc-tag was also performed in cells without tag.

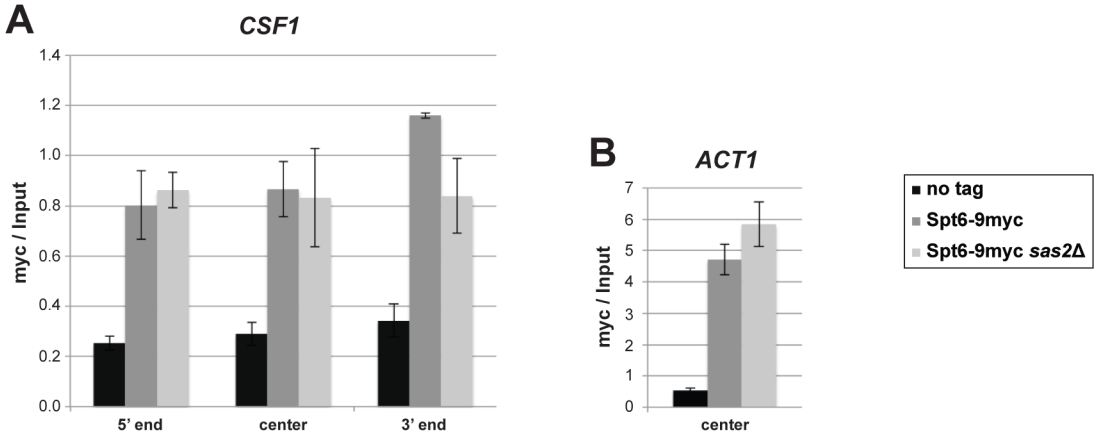

Figure 15. Spt6-9myc was highly enriched at the highly expressed *ACT1* gene and less enriched at the poorly expressed *CSF1* gene.
ChIP against myc-tag was performed in cells expressing Spt6-9myc in wild type (AEY5486) as well as *sas2*Δ background (AEY5487) and as control in a strain with no myc-tag (AEY2). Spt6-9myc levels were quantified at the poorly expressed *CSF1* gene (**A**) and at the highly expressed *ACT1* gene (**B**). (**A**) Spt6-9myc was moderately enriched at *CSF1* in wild type as well as *sas2*Δ background. At the 3' end of the *CSF1* ORF, Spt6-9myc levels were reduced upon deletion of *SAS2*. (**B**) Spt6-9myc was highly enriched at *ACT1* in wild type as well as *sas2*Δ background. Error bars represent SD of three biological replicates.

As expected from earlier work (Ivanovska et al. 2011), Spt6-9myc was moderately enriched at the poorly expressed *CSF1* gene (Figure 15A) and strongly associated with the highly expressed *ACT1* gene (Figure 15B). This enrichment of Spt6-9myc was independent of Sas2-mediated H4 K16 acetylation, because wild type and *sas2*Δ cells showed similar levels of Spt6-9myc enrichment with the only exception of the 3' end of the *CSF1* ORF (Figure 15A). This difference in chromatin association of Spt6-9myc at the 3' end of *CSF1* suggests that Spt6 activity at 3' ends of poorly expressed genes is to some extent facilitated by Sas2-mediated H4 K16 acetylation. However, this effect was rather mild and therefore was not pursued.

So far, as observed in previous studies, association of Spt6 with genes correlated with their expression level, with a high Spt6 level at highly expressed genes and less Spt6 at poorly transcribed genes. It remained to be addressed whether this difference in chromatin association of Spt6 depending on a gene's expression level could also be observed at *GAL10* upon its repression. In order to address this, cells expressing Spt6-9myc in wild type background were shifted from galactose to glucose, and Spt6-9myc association at *GAL10* was measured by ChIP.

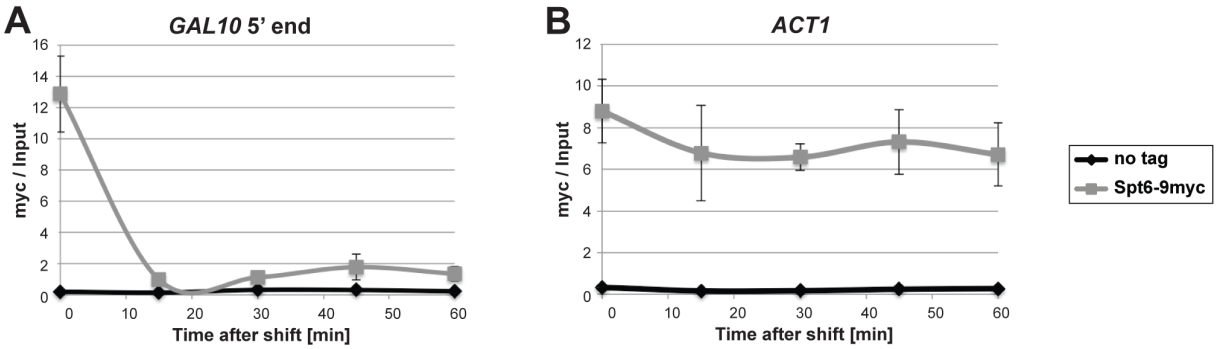

Figure 16. Upon shift from galactose to glucose, Spt6-9myc vanished from the *GAL10* gene due to its repression, whereas Spt6-9myc levels at *ACT1* were not influenced.
Cells expressing Spt6-9myc (AEY5486) were shifted from galactose- to glucose-containing medium to repress *GAL10* expression. Spt6-9myc association to *GAL10* (**A**) and *ACT1* (**B**) was measured by ChIP. An equally treated strain bearing no myc-tag (AEY2) served as control. (**A**) Spt6-9myc was highly enriched at *GAL10* in galactose medium (time point "0"), whereas upon shift to glucose, Spt6-9myc association decreased to background level within 15 min. (**B**) Spt6-9myc levels at *ACT1* were similar in galactose- and glucose-containing medium, showing that medium shift had no influence on transcription of *ACT1*. Error bars represent SD of three biological replicates.

Importantly, Spt6-9myc levels at *GAL10* were high in medium containing galactose (Figure 16A, time point "0") and decreased to background levels upon shift to glucose during the repression of *GAL10*. At *ACT1*, Spt6-9myc levels did not change upon shift of the medium (Figure 16B), which was in agreement with the notion that *ACT1* transcription was not influenced by the shift of the medium.

In summary, Spt6 could not be shown to be involved in the incorporation of Sas2-mediated H4 K16Ac in a direct manner. Upon repression, no enrichment of H4 K16Ac at *GAL10* was observed in *spt6-1004* cells. In fact, H4 K16Ac levels in *spt6-1004* cells were higher than in wild type cells at all investigated genes independent of their expression level. Interestingly, it could be shown that in *spt6-1004* cells H4 occupancy was decreased compared to wild type cells, especially at the center and 3' ends of ORFs. Furthermore, it could also be observed that in *spt6-1004* cells *GAL10* was not completely repressed in medium containing glucose. These results suggested that Spt6 is involved in the incorporation of K16-unacetylated H4 during transcription, thereby influencing H4 K16Ac levels in an indirect manner. This is in agreement with earlier observations of a role for Spt6 in transcription-coupled histone deposition (Kaplan et al. 2003).

3.2.3 Influences of the histone chaperone Rtt106 on H4 K16Ac during gene repression

Rtt106 is a histone chaperone that together with CAF-1 loads H3/H4-tetramers onto newly replicated DNA as a first step of nucleosome assembly (Huang et al. 2005). In addition to this replication-coupled function of Rtt106 in nucleosome assembly, Rtt106 is also know to be

involved in the regulation of chromatin structure during transcription elongation. In this context, Rtt106 was shown to be associated with actively transcribed genes (Imbeault et al. 2008).

Initially, we sought to address which histone chaperones influence the incorporation of H4 K16Ac into chromatin during transcriptional repression. In G1-arrested cells, a deletion of *RTT106* caused a slightly reduced enrichment of H4 K16Ac at the *GAL10* gene at the later time points of transcriptional repression of this gene compared to wild type cells (Figure 11D). This finding suggested a role for Rtt106 in the incorporation of H4 K16Ac into chromatin upon gene repression. To investigate the influence of Rtt106 on H4 K16Ac in more detail, in a first experiment it was addressed whether, upon deletion of *RTT106*, there was also a reduced enrichment of H4 K16Ac at *GAL10* upon its repression in an asynchronous culture. Rather than in G1-arrested cells as in the previous experiment, this would indicate a cell-cycle independent function for Rtt106 in the incorporation of H4 K16Ac during transcriptional repression. In order to address this, asynchronous cultures of wild type, *sas2*Δ and *rtt106*Δ cells in galactose-containing medium were shifted to medium containing glucose. Samples of cells for ChIP were taken at regular time points, in which at time point "0" cells were taken from galactose-containing culture and the following samples of cells were taken from cultures growing on glucose. ChIP was performed against H4 K16Ac and against unmodified histone H4 for normalization.

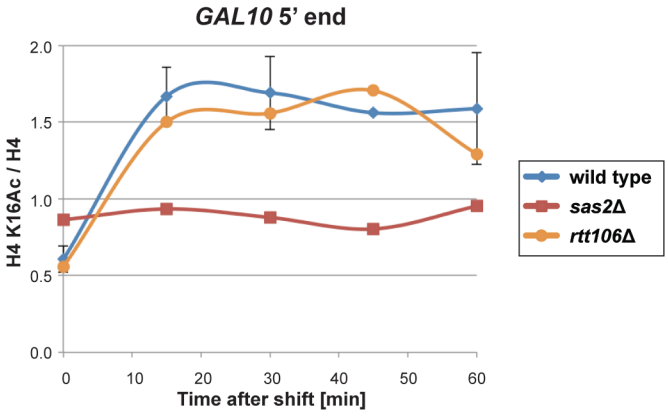

Figure 17. A deletion of *RTT106* had no effect on the enrichment of H4 K16Ac during transcriptional repression of *GAL10* in asynchronous cultures.
Asynchronous cultures of wild type, *sas2*Δ and *rtt106*Δ cells were shifted from galactose- (time point "0") to glucose-containing medium. H4 K16Ac levels at the 5' end of the *GAL10* ORF were analyzed by ChIP. H4 K16Ac/H4-values were normalized to H4 K16Ac/H4-values at *ACT1* at the respective time points. Error bars for wild type cells represent SD of three biological replicates. For *sas2*Δ and *rtt106*Δ the experiment was done once. *rtt106*Δ cells did not show a reduction of H4 K16 acetylation compared to wild type cells.

When *GAL10* was repressed in an asynchronous culture of *rtt106*Δ cells, the incorporation of H4 K16Ac into chromatin at *GAL10* was indistinguishable from that of a wild type strain

(Figure 17). If not normalized to relative H4 K16Ac values at *ACT1*, *rtt106*Δ cells also showed levels of H4 K16Ac comparable to wild type cells (data not shown). Thus, the decreased enrichment of H4 K16 acetylation at *GAL10* in G1-arrested *rtt106*Δ cells could not be confirmed in an asynchronous culture, suggesting that Rtt106 might not influence H4 K16Ac incorporation upon gene repression.

We furthermore tested the eventuality that the observed effect of a deletion of *RTT106* on H4 K16Ac enrichment at the repressed *GAL10* gene could be a consequence of the cell-cycle arrest in G1-phase (Figure 11D). In order to address this, wild type, *sas2*Δ and *rtt106*Δ cells were arrested in G1-phase in standard full-medium containing glucose. Cell samples for ChIP against H4 K16Ac and against unmodified histone H4 for normalization were taken. For comparison, H4 K16Ac levels were measured at the poorly expressed *CSF1* gene as well as at the highly expressed *ACT1* gene.

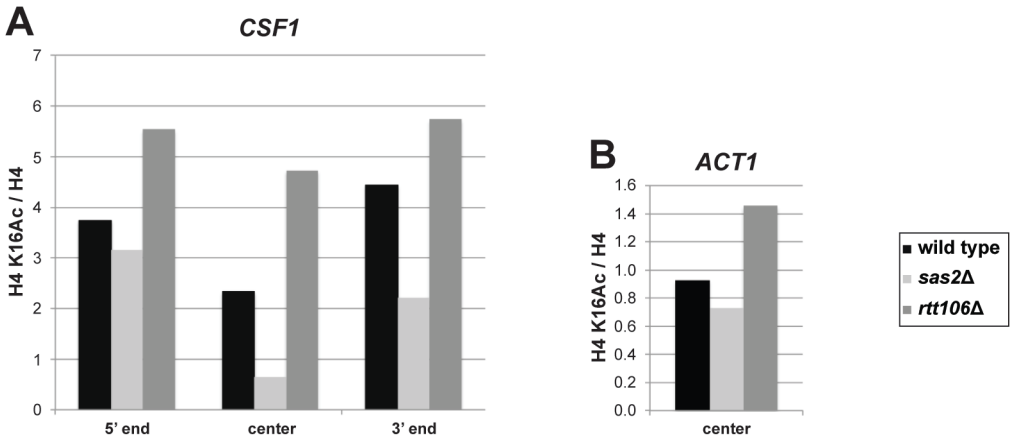

Figure 18. H4 K16Ac levels of G1-arrested *rtt106*Δ cells were higher than H4 K16Ac levels of wild type and *sas2*Δ cells.
G1-arrested *rtt106*Δ cells had higher levels of acetylated H4 K16 at the poorly expressed *CSF1* gene (**A**) as well as at the highly expressed *ACT1* (**B**) gene when compared to wild type cells. Experiment was done once.

Upon arrest in G1-phase, levels of acetylated H4 K16Ac were not reduced, but rather slightly increased, in *rtt106*Δ cells compared to wild type cells at *CSF1* (Figure 18A) and *ACT1* (Figure 18B). However, with the experiments conducted so far, no explanation for the observed reduction of H4 K16Ac at *GAL10* during its repression in G1-arrested *rtt106*Δ cells compared to wild type cells could be found. Apparently, the effect of a deletion of *RTT106* on H4 K16Ac incorporation during repression in G1-arrested cells might not have been statistically significant.

In a next attempt to investigate the potential effects of a deletion of *RTT106* on H4 K16Ac, levels of H4 K16Ac on bulk histone H4 were measured by Western blotting in whole cell extracts of *rtt106*Δ and *rtt106*Δ *sas2*Δ cells and were compared to wild type and *sas2*Δ cells.

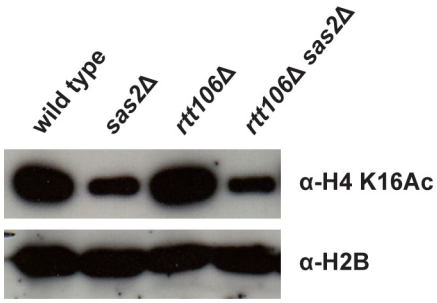

Figure 19. Deletion of *RTT106* did not change H4 K16 acetylation levels in whole cell extracts compared to wild type.
Whole cell extracts of wild type, *sas2*Δ, *rtt106*Δ and *rtt106*Δ *sas2*Δ were subjected to Western blot for acetylated H4 K16. As expected, *sas2*Δ cells showed a markedly reduced H4 K16Ac level compared to wild type cells, whereas a deletion of *RTT106* did not further reduce amounts of acetylated H4 K16 compared to wild type or *sas2*Δ cells, respectively.

The deletion of *RTT106* did not alter H4 K16Ac levels on bulk histones compared to wild type cells (Figure 19). Furthermore, the reduced level of H4 K16Ac in *sas2*Δ cells was unaffected by the deletion of *RTT106*. If Rtt106 is involved in the incorporation of H4 K16Ac, then one might expect that upon deletion of *RTT106*, H4 K16Ac on bulk histones would be reduced. However, *rtt106*Δ did not influence H4 K16 acetylation on bulk histones. Interestingly, Rtt106 has previously been shown to be associated with actively transcribed genes (Imbeault et al. 2008), which is difficult to reconcile with our observation that Rtt106 might be involved in the gene repression-associated incorporation of H4 K16Ac. Therefore, we tested the physical association of Rtt106 at actively transcribed or repressed genes by ChIP.

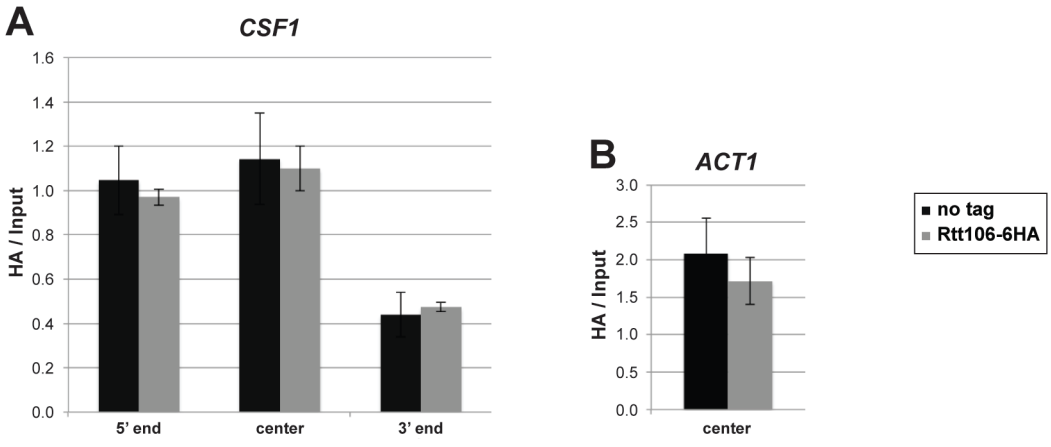

Figure 20. Rtt106-6HA could not be immunoprecipitated.
Compared to a no-tag control, Rtt106-6HA could not be immunoprecitpitated neither at the poorly transcribed *CSF1* gene (**A**), nor at the highly transcribed *ACT1* (**B**) gene. Error bars represent SD of three biological replicates.

Contrary to our expectation, a 6xHA-tagged version of Rtt106 was not enriched at three positions within the poorly transcribed *CSF1* gene (Figure 20A), nor at the highly expressed

ACT1 gene (Figure 20B). Moreover, Rtt106-6HA was also not associated with two other genes tested, *VPS15* and *RIP1* (data not shown).

According to Imbeault et al. (2008), Rtt106 should be associated with *ACT1*. We therefore hypothesized that our inability to ChIP Rtt106 at *ACT1* might be caused by a reduced functionality of Rtt106-6HA due to the C-terminal tag used here. Imbeault et al. (2008) used a myc-tagged version of Rtt106 (Rtt106-13myc) for der ChIP experiments. However, using a strain with a C-terminally 9myc-tagged version of Rtt106 for ChIP, Rtt106-9myc could not be immunoprecipitated over background level (no-tag control) neither at the poorly transcribed *CSF1* gene (Figure 21A), nor at the highly expressed *ACT1* gene (Figure 21B). Again, at two other genes tested, *VPS15* and *RIP1*, Rtt106-9myc could also not be precipitated over background levels (data not shown).

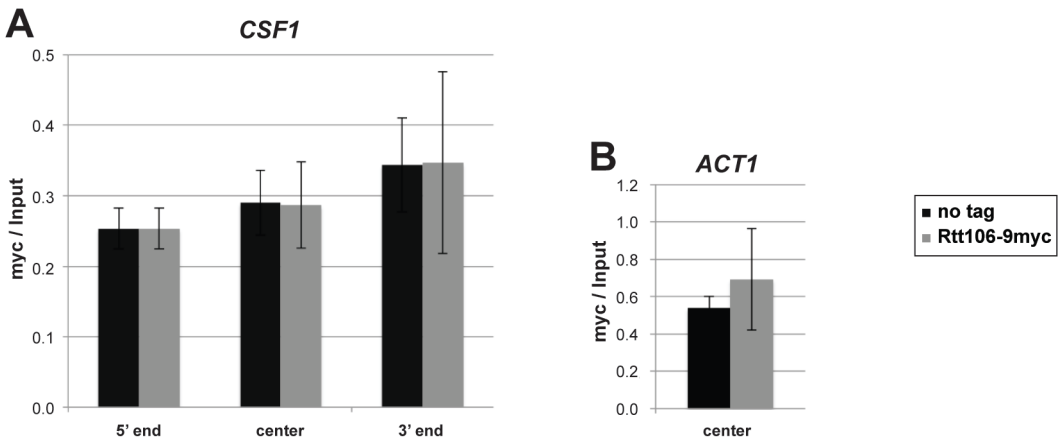

Figure 21. Rtt106-9myc could also not be immunoprecipitated.
Compared to a no-tag control, Rtt106-9myc could not be immunoprecitpitated neither at the poorly transcribed *CSF1* gene (**A**), nor at the highly transcribed *ACT1* (**B**) gene. Error bars represent SD of three biological replicates.

In a final attempt to immunoprecipitate Rtt106-9myc, cells were shifted from medium containing galactose to medium containing glucose. Rtt106-9myc association to the *GAL10* gene was monitored while it was actively transcribed (time point "0") and repressed (15 to 60 min after the shift to glucose). As a control, the same time course experiment was performed with a strain without a myc-tag.

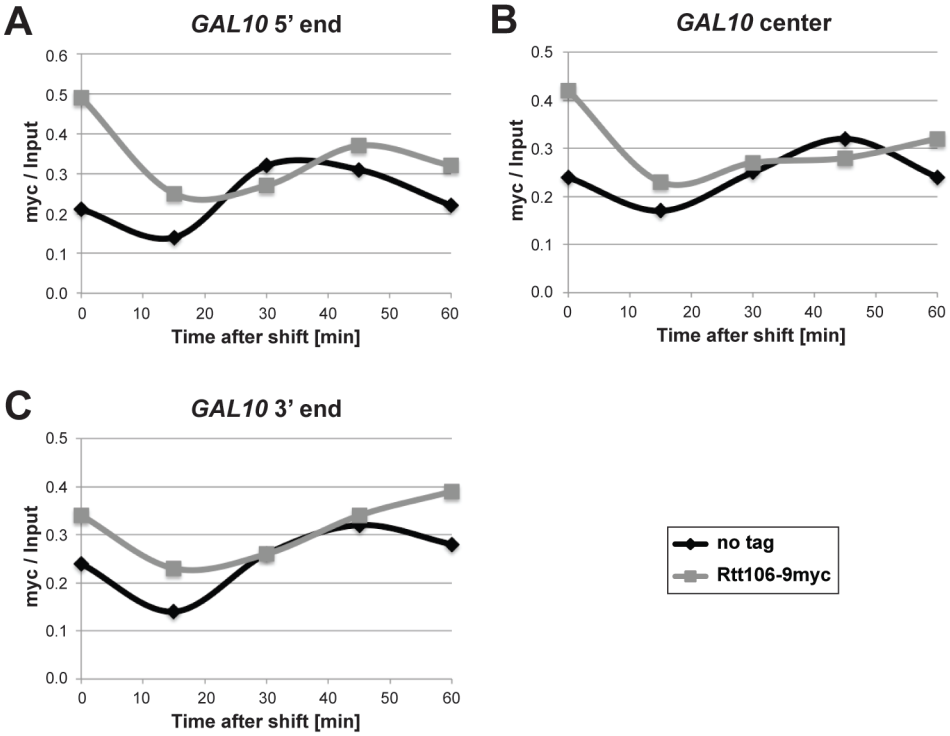

Figure 22. Slight enrichment of Rtt106-9myc at *GAL10* during active transcription.
When cultured in medium containing galactose (time point "0"), Rtt106-9myc was slightly enriched at the 5' end (**A**), the center (**B**) and the 3' end (**C**) of the *GAL10* ORF. Upon shift to medium containing glucose, the Rtt106-9myc signal dropped to background levels (no-tag control) at all three investigated loci. The experiment was done once.

Cells cultured in medium containing galactose (Figure 22, time point "0"), showed a slight enrichment of Rtt106-9myc at the 5' end (Figure 22A), the center (Figure 22B) and the 3' end (Figure 22C) of the *GAL10* ORF. Upon shifting the cells to glucose, Rtt106-9myc enrichment decreased to background levels. These results are suggestive of an enrichment of Rtt106 at actively transcribed genes, as seen by Imbeault et al. (2008). Nevertheless, this enrichment of Rtt106 at the actively transcribed *GAL10* in our own ChIP experiments was very mild, and no Rtt106 enrichment was seen at the highly transcribed *ACT1* gene. Taken together, we conclude that under the experimental conditions used here, Rtt106 cannot be immunoprecipitated neither at highly, nor at poorly expressed or repressed genes.

Based on the results of Imbeault et al. (2008), which showed a function for Rtt106 in the regulation of chromatin structure during transcription elongation, our hypothesis that Rtt106 is involved in the incorporation of acetylated H4 K16 during gene repression (Figure 11D) was counterintuitive. In a last approach to study the influence of Rtt106 on Sas2-mediated H4 K1Ac, we asked whether Rtt106 and Sas2 might physically interact with each other. In order to address this question, coimmunoprecipitation experiments (CoIP) were performed with cells expressing Rtt106-6HA and simultaneously overexpressing Sas2. CoIP was

performed with a α-Sas2 antibody (Meijsing and Ehrenhofer-Murray 2001) or with a α-HA antibody, respectively.

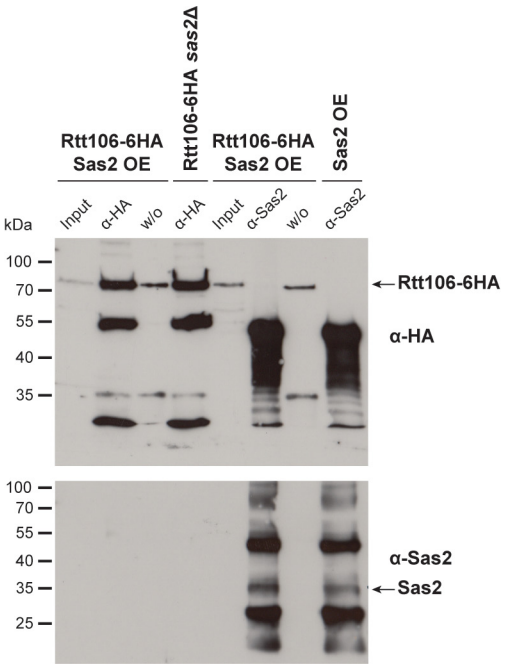

Figure 23. Rtt106-6HA and Sas2 did not show a physical interaction in coimmunoprecipitation experiments.
Coimmunoprecipitation experiments in lysates of cells expressing Rtt106-6HA and overexpressing Sas2 from a 2μ-plasmid (Sas2 OE) against HA or Sas2, respectively. As negative controls, IPs against HA in Rtt106-6HA *sas2*Δ cells and against Sas2 in Sas2 overexpressing cells were included, as well as lysates treated without antibody (w/o). Rtt106-6HA could not be immunoprecipitated using the α-Sas2 antibody. *Vice versa*, Sas2 was not immunoprecipitated using the α-HA antibody. The experiment was repeated several times using different conditions. Western blots of a representative experiment are shown.

In the CoIP experiments, no physical interaction between Rtt106 and Sas2 could be detected (Figure 23), regardless of the experimental conditions for the IP that were applied.

Summarizing the experimental results presented here, Rtt106 did not influence Sas2-mediated H4 K16 acetylation. In the initial experiment, compared to wild type cells, a deletion of *RTT106* caused a mild reduction of enrichment of acetylated H4 K16 at *GAL10* when it became repressed in the G1-phase of the cell cycle. This reduced enrichment of H4 K16Ac upon deletion of *RTT106* could not be seen in asynchronous cultures, and a G1-arrest alone did not influence H4 K16Ac levels at highly and poorly expressed genes in *rtt106*Δ cells. Upon deletion of *RTT106*, acetylation of K16 of bulk histone H4 did not change. Furthermore, Rtt106 could not be found in association with actively transcribed genes as described by Imbeault et al. (2008). Finally, no physical interaction between Rtt106 and Sas2 could be observed. These results suggested that Rtt106 does not influence Sas2-mediated H4 K16 acetylation. The observed effect of a deletion of *RTT106* on the enrichment of

H4 K16Ac at repressed genes in G1-phase might therefore not be statistically significant, thus indicating that the influence of Rtt106 on H4 K16Ac, if any, is only minor.

3.2.4 Genome-wide influences of the histone deacetylase Sir2 on H4 K16Ac levels at euchromatic genes

The previous chapters addressed when Sas2-mediated H4 K16Ac is incorporated into chromatin and how this incorporation is influenced by histone chaperones. However, the function of H4 K16Ac in euchromatic regions in addition to its boundary function to prevent heterochromatic SIR spreading remained unaddressed. In this section, the hypothesized function of H4 K16Ac in preventing the spurious binding of the SIR complex to euchromatic genes was investigated in more detail.

Sas2-mediated H4 K16Ac prevents the excessive spreading of SIR-mediated heterochromatin into euchromatic regions, e.g. at telomeres (Kimura et al. 2002; Suka et al. 2002). Next to this function in the maintenance of euchromatic identity at heterochromatin-proximal loci, H4 K16Ac was found to be slightly inhibitory to RNA Pol II-mediated transcription (Heise 2011; Heise et al. 2012). In *sas2*Δ cells, H4 K16Ac levels are decreased within the majority of open reading frames, whereas there is only little change of H4 K16Ac in intergenic regions. Furthermore, regions of low exchange of histone H3, representing poorly transcribed genes, show the most pronounced loss of H4 K16Ac upon deletion of *SAS2*. In line with this, H4 K16Ac levels at poorly transcribed genes in wild type cells are higher than H4 K16Ac levels at highly expressed genes (which could also be seen in experiments presented above, e.g. Figure 10A). Based on these findings, one hypothesis describing the genome-wide function of Sas2-catalyzed H4 K16Ac is that this histone mark protects euchromatic regions from being spuriously bound by SIR proteins. Thus, H4 K16Ac is thought to keep chromatin amenable to transcription.

By ChIP-chip experiments in *sas2*Δ cells, H4 K16Ac levels were shown to be reduced especially at the center and the 3' end of ORFs of long, poorly transcribed genes (Heise 2011; Heise et al. 2012). This raised the question whether this decrease of H4 K16Ac in euchromatin was due to the absence of Sas2 or due to an activity of the histone deacetylase Sir2 outside of heterochromatin. At subtelomeric regions, a deletion of *SAS2* causes the excessive spreading of SIR proteins into adjacent euchromatic regions leading to further Sir2-mediated deacetylation of H4 K16, and thereby transcriptionally silencing these loci (Kimura et al. 2002; Suka et al. 2002). In order to address whether H4 K16Ac at euchromatic regions in *sas2*Δ cells is also reduced by Sir2-mediated histone deacetylation, chromatin

immunoprecipitation against H4 K16Ac and, for normalization, against unmodified histone H4 was performed in *sir2*Δ and *sir2*Δ *sas2*Δ cells. The immunoprecipitated DNA was then hybridized to high-resolution tiling arrays (ChIP-chip). The tiling arrays used in this experiment comprised 25-mer oligonucleotides with a 5 bp resolution (Affymetrix), thus tiling the complete genome of *S. cerevisiae*. For data analysis, ChIP-chip results of wild type and *sas2*Δ cells obtained by Franziska Heise (Heise 2011; Heise et al. 2012) were used.

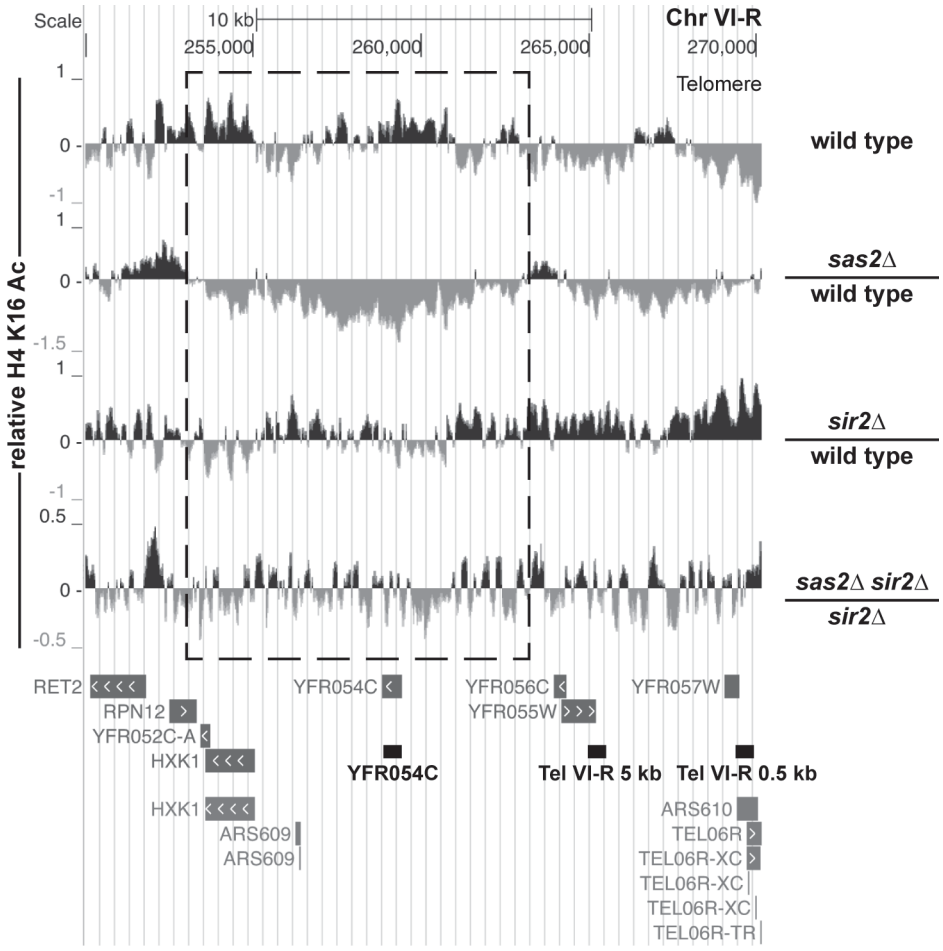

Figure 24. At telomere-distal loci, H4 K16Ac is reduced in *sas2*Δ cells due to the excessive spreading of the SIR complex into this region.
ChIP-chip data for each strain was normalized as described above (Figure 7). H4 K16Ac levels at telomeric and subtelomeric regions of the right arm of chromosome VI are shown for wild type cells (AEY1, first row) as well as for *sas2*Δ cells (AEY269) compared to wild type (second row), *sir2*Δ cells (AEY2582) compared to wild type (third row) and *sas2*Δ *sir2*Δ cells (AEY5073) compared to *sir2*Δ (forth row). In wild type cells, H4 K16Ac was decreased at telomere-proximal loci and more or less increased with increasing distance from the telomere. In *sas2*Δ cells, H4 K16Ac was markedly reduced at a large subtelomeric region (marked by dashed box) compared to wild type. Upon deletion of *SIR2*, H4 K16Ac levels were increased at telomere-proximal loci compared to wild type cells. Upon deletion of *SAS2* in *sir2*Δ background, H4 K16Ac levels at the subtelomeric region (marked by dashed box) were not further decreased compared to *sir2*Δ cells. Below, the localization of loci analyzed in following ChIP-qPCR experiments ("YFR054C", "Tel VI-R 5 kb" and "Tel VI-R 0.5 kb") is depicted. Bioinformatic data analysis was performed by A. E. Ehrenhofer-Murray.

To evaluate the results of the ChIP-chip experiments in *sir2*Δ and *sir2*Δ *sas2*Δ cells, H4 K16Ac levels were investigated at the telomeric and subtelomeric regions of the right arm

of chromosome VI (Figure 24), where effects of a deletion of *SAS2* or *SIR2* are well-studied (Kimura et al. 2002; Suka et al. 2002; Heise et al. 2012). In wild type cells, H4 K16Ac was markedly reduced at the telomere-proximal region compared to the mean H4 K16Ac level over the whole genome. This low level of H4 K16Ac was a consequence of the HDAC activity of Sir2, a component of the heterochromatic SIR complex, in this region. The SIR complex was prevented to spread into subtelomeric euchromatic regions (marked by the dashed box in Figure 24), by Sas2-catalyzed H4 K16Ac, which was increased in this region. Upon deletion of *SAS2*, H4 K16Ac levels decreased at this subtelomeric region compared to wild type cells (Figure 24, second row). As has previously been shown (Kimura et al. 2002), this decrease in H4 K16Ac is caused by the excessive spreading of the SIR complex, including Sir2, into this subtelomeric region because of the lack of Sas2-mediated H4 K16Ac. This consequence of a deletion of *SAS2* could be confirmed with the ChIP-chip experiments presented here: As expected, upon deletion of *SIR2*, H4 K16Ac levels were increased at telomere-proximal loci, whereas H4 K16Ac levels at subtelomeric loci remained unaffected (Figure 24, third row). If the decrease of H4 K16Ac in subtelomeric regions in *sas2*Δ cells would be caused by the loss in Sas2 activity, then a deletion of *SAS2* in *sir2*Δ cells would cause a decrease of H4 K16Ac levels compared to *sir2*Δ cells. However, upon deletion of *SAS2* in *sir2*Δ background, H4 K16Ac levels at the subtelomeric region were not further decreased compared to *sir2*Δ cells (Figure 24, forth row). Thus, it could be concluded that due to the loss of boundary function of H4 K16Ac in *sas2*Δ cells, the SIR complex excessively spreads into subtelomeric regions, where Sir2 deacetylates H4 K16Ac.

Due to the fact that H4 K16Ac levels for each locus were averaged to the mean H4 K16Ac level over the whole genome in the respective strain, i.e. relative H4 K16Ac values were given, this experiment did not give information about the absolute H4 K16Ac levels at the investigated loci. Therefore, absolute H4 K16Ac levels were measured by conventional ChIP in wild type, *sas2*Δ, *sir2*Δ and *sir2*Δ *sas2*Δ cells at telomeric and subtelomeric loci. ChIP against H4 K16Ac and against unmodified histone H4 for normalization was performed in asynchronous cultures of the respective strains. Additionally, ChIP was also performed in *sir3*Δ and *sir3*Δ *sas2*Δ cells to address whether the whole SIR complex consisting of Sir2/3/4 excessively spread into subtelomeric regions. H4 K16Ac levels were quantified at three telomeric and subtelomeric loci (localization marked in Figure 24).

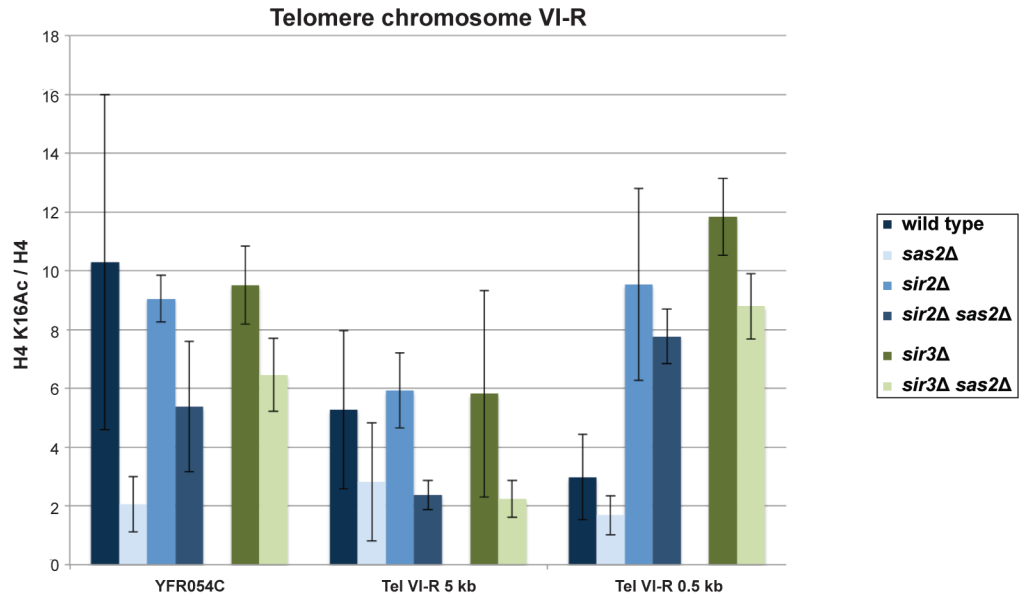

Figure 25. Decrease of H4 K16Ac in s*as2*Δ cells at the subtelomeric gene *YFR054C* is due to an excessive spreading of the SIR complex.
ChIP against H4 K16Ac was performed in asynchronous cultures of wild type (AEY1), *sas2*Δ (AEY269), *sir2*Δ (AEY2582), *sir2*Δ *sas2*Δ (AEY5073), *sir3*Δ (AEY19) and *sir3*Δ *sas2*Δ (AEY4658) cells. H4 K16Ac levels normalized to unmodified histone H4 were measured by qPCR at three telomeric or subtelomeric loci at the right arm of chromosome VI: *YFR054C*, Tel VI-R 5 kb, Tel VI-R 0.5 kb (localization of the investigated loci is marked in Figure 24). In wild type cells, H4 K16Ac decreased with decreasing distance to the telomere. Upon deletion of *SAS2*, H4 K16Ac was reduced at all three investigated loci. In *sir2*Δ cells, H4 K16Ac levels were as high as subtelomeric wild type levels. In *sir2*Δ *sas2*Δ cells, H4 K16Ac was increased at *YFR054C* compared to wild type cells, which hint at a spreading of Sir2 into this subtelomeric region upon deletion of *SAS2*. The whole SIR complex spread into subtelomeric regions upon deletion of *SAS2*, because deletion of *SIR3* in *sas2*Δ background caused an increase of H4 K16Ac at *YFR054C* as it was seen for *sir2*Δ *sas2*Δ cells. Error bars represent SD of three biological replicates.

In wild type cells, H4 K16Ac decreased with decreasing distance from the telomere (Figure 25). Upon deletion of *SAS2*, H4 K16Ac levels were low at telomeric as well as subtelomeric loci. This decrease of H4 K16Ac at *YFR054C* in *sas2*Δ cells could be suppressed by deleting *SIR2* in s*as2*Δ background. This suggested that in *sas2*Δ cells, the SIR complex spread into subtelomeric regions due to a lack of Sas2-mediated H4 K16Ac functioning as a boundary element for SIR spreading. In *sir3*Δ *sas2*Δ cells, an increase of H4 K16Ac compared to *sas2*Δ cells was observed, which confirmed that the whole SIR complex spread into subtelomeric regions upon deletion of *SAS2*. Collectively, these results confirmed the tightly regulated equilibrium of hypoacetylation at telomeric and hyperacetylation at subtelomeric regions.
The decrease of H4 K16Ac observed at long, poorly transcribed ORFs in *sas2*Δ cells (Heise 2011; Heise et al. 2012) could be due to the loss of Sas2 activity or, alternatively, due to an HDAC activity of Sir2 in euchromatic regions. In order to address this, we took advantage of our ChIP-chip data against H4 K16Ac in *sir2*Δ and *sir2*Δ *sas2*Δ cells.

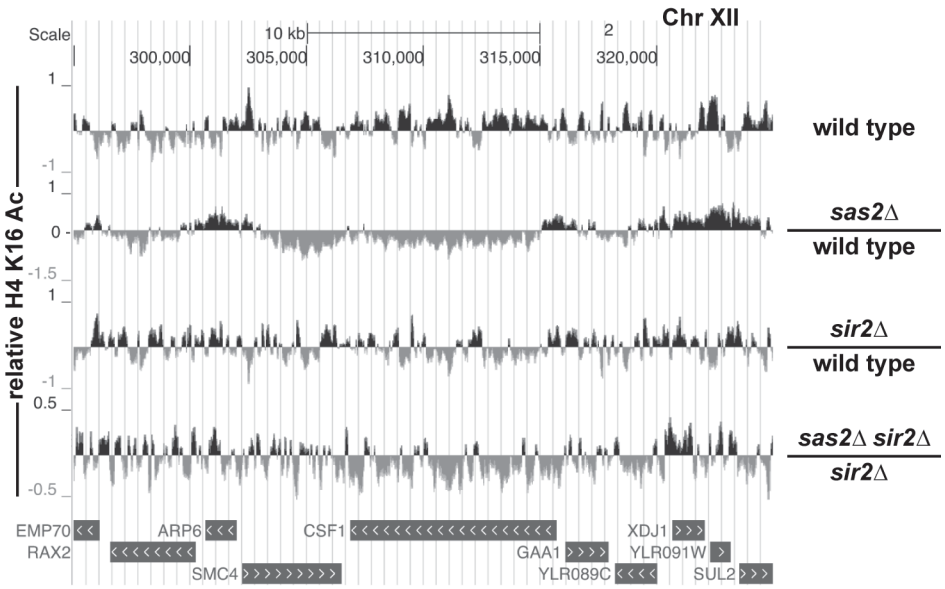

Figure 26. Upon deletion of *SAS2*, H4 K16Ac is decreased at long, poorly-transcribed genes, e.g. at *CSF1* and *SMC4* on chromosome XII, compared to wild type, which was not due to Sir2-mediated histone deacetylation at these genes.
ChIP-chip data for each strain was normalized as described above (Figure 7). H4 K16Ac levels at a typical region of chromosome XII are shown for wild type cells (AEY1, first row) as well as for *sas2*Δ cells (AEY269) compared to wild type (second row), *sir2*Δ cells (AEY2582) compared to wild type (third row) and *sas2*Δ *sir2*Δ cells (AEY5073) compared to *sir2*Δ (forth row). At *CSF1* and *SMC4*, two long, poorly transcribed genes, H4 K16Ac levels were decreased in *sas2*Δ compared to wild type cells. This decrease of H4 K16Ac could not be counteracted by additional deletion of *SIR2* in *sas2*Δ background, which suggested that the HDAC Sir2 was not active in this euchromatic region of the genome.

As has been shown (Heise 2011; Heise et al. 2012), H4 K16Ac levels were decreased at long, poorly transcribed genes, e.g. at *CSF1* and *SMC4*, in *sas2*Δ cells compared to wild type (Figure 26, second row). To determine the cause of this decrease, H4 K16Ac levels were investigated upon deletion of *SAS2* in s*ir2*Δ background compared to H4 K16Ac levels in *sir2*Δ cells alone (Figure 26, forth row). If the decrease of H4 K16Ac at *CSF1* and *SMC4* in *sas2*Δ cells would be caused by the loss in Sas2 activity, then a deletion of *SAS2* in *sir2*Δ cells would cause a decrease of H4 K16Ac levels compared to *sir2*Δ cells. In deed, in *sas2*Δ *sir2*Δ cells, H4 K16Ac levels were decreased compared to *sir2*Δ cells, indicating that the deletion of *SAS2* caused a reduction of H4 K16Ac levels, regardless of whether Sir2 was present or not. This effect was also observed at other long, less transcribed genes throughout the genome (data not shown). As expected, upon deletion of *SIR2* alone, H4 K16Ac levels did not markedly change compared to wild type (Figure 26, third row).

The decrease of H4 K16Ac levels seen in *sas2*Δ *sir2*Δ cells compared to *sir2*Δ cells at long, poorly transcribed genes suggested no HDAC activity of Sir2 at these genes, but instead, that the decrease at these genes was due to a loss of Sas2 activity in *sas2*Δ cells. Nevertheless, this effect could be due to the normalization of the ChIP-chip data to the mean H4 K16Ac level over the whole genome of the respective strains (as explained above). Thus, these results were

verified by conventional ChIP. For this purpose, ChIP against H4 K16Ac and, for normalization, against unmodified histone H4 was performed in asynchronous cultures of wild type, *sas2*Δ, *sir2*Δ as well as *sir2*Δ *sas2*Δ cells. H4 K16Ac levels were quantified at *CSF1* (Figure 27) and *SMC4* (data not shown).

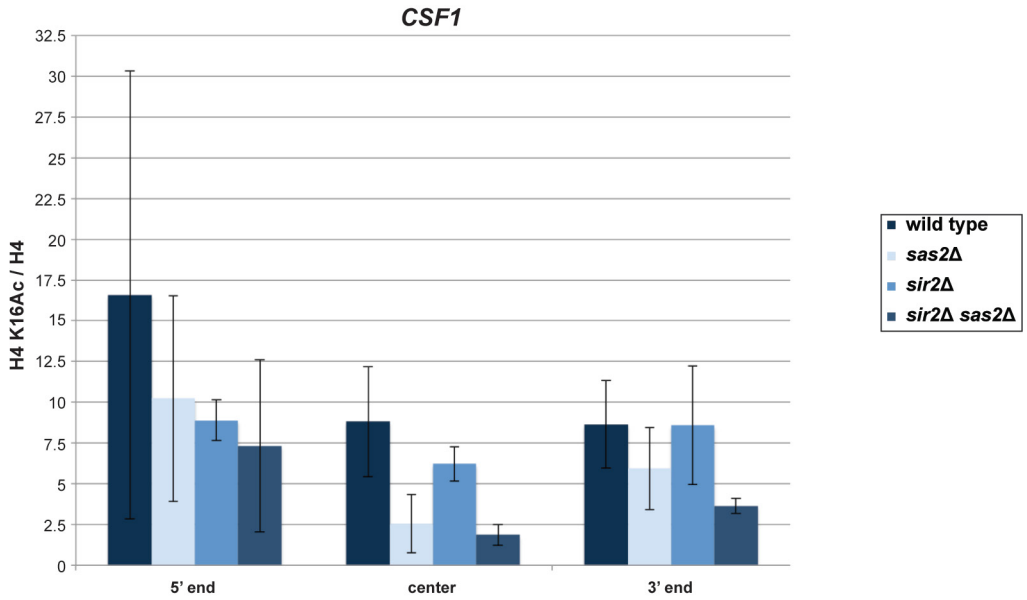

Figure 27. The decrease of H4 K16Ac at the center and the 3' end of the ORF of *CSF1* in *sas2*Δ cells was not due to a Sir2-mediated deacetylation activity.
ChIP against H4 K16Ac was performed in asynchronous cultures of wild type (AEY1), *sas2*Δ (AEY269), *sir2*Δ (AEY2582) and *sir2*Δ *sas2*Δ (AEY5073) cells. H4 K16Ac levels normalized to unmodified histone H4 were measured by qPCR at three loci at *CSF1*. In *sas2*Δ cells, H4 K16Ac was decreased at the center and the 3' end of the ORF of *CSF1* compared to wild type cells. In *sir2*Δ *sas2*Δ cells, there was no increase of H4 K16Ac compared to *sas2*Δ cells. The deletion of *SIR2* did not influence H4 K16Ac levels at the center and the 3' end of *CSF1*. These results suggested, that Sir2 did not influence H4 K16Ac levels at long, poorly transcribed genes. Error bars represent SD of three biological replicates.

As expected, at the center and the 3' end of the *CSF1* gene, H4 K16Ac levels were reduced in *sas2*Δ cells compared to wild type cells (Figure 27). Upon deletion of *SIR2* in *sas2*Δ background, H4 K16Ac levels remained low, comparable to *sas2*Δ cells. The deletions of *SIR2* alone had no effect on H4 K16Ac levels at the center and 3' end of *CSF1* compared to wild type cells. Similar results could also be obtained for the *SMC4* gene (data not shown). The ChIP-qPCR results presented here confirmed that the decrease of H4 K16Ac at long, poorly transcribed genes in *sas2*Δ was not due to a Sir2-mediated deacetylation, but rather was a consequence of the lacking activity of Sas2.

In order to exclude any Sir2-mediated deacetylation at *CSF1* and other poorly transcribed genes, the association of Sir2 with *CSF1* was measured by ChIP in cells expressing a 9myc-tagged version of Sir2 in wild type as well as *sas2*Δ background, respectively. As a control, ChIP against the myc-tag was performed in cells without tag. Sir2-9myc binding to chromatin

was measured by qPCR at *CSF1* and, as a control, at telomeric and subtelomeric loci of the right arm of chromosome VI.

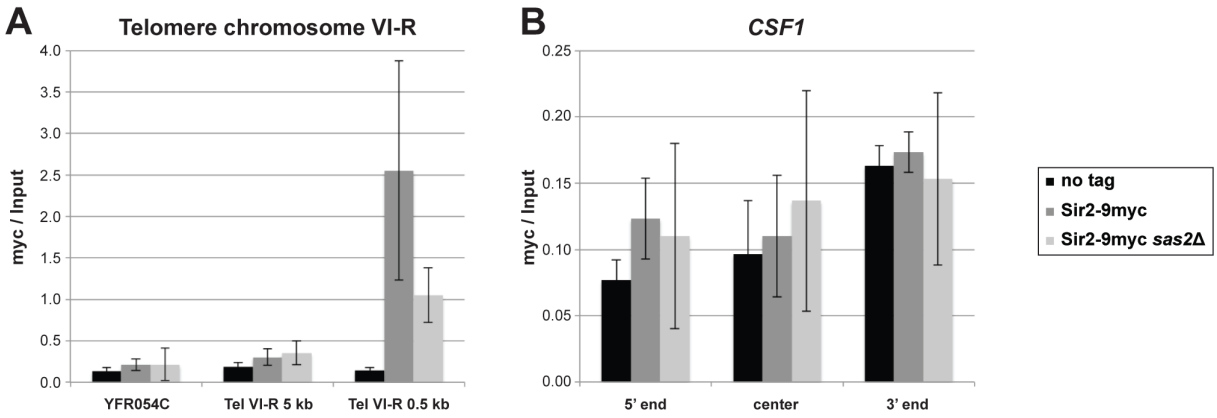

Figure 28. Sir2-9myc was enriched at telomeric chromatin at the right arm of chromosome VI, whereas no Sir2-9myc was associated with the *CSF1* gene.
ChIP against myc-tag was performed in cells expressing Sir2-9myc in wild type (AEY2554) as well as *sas2*Δ background (AEY5289) and as control in a strain expressing no myc-tag (AEY1558). Sir2-9myc levels were quantified at telomeric and subtelomeric loci at the right arm of chromosome VI (**A,** localization of the investigated loci is marked in Figure 24) and at the poorly expressed *CSF1* gene (**B**). (**A**) Sir2-9myc was highly enriched at the telomere-proximal locus Tel VI-R 0.5 kb in wild type cells, whereas there was less Sir2-9myc bound at this locus in *sas2*Δ background. At the more telomere-distal loci Tel VI-R 5 kb and *YFR054C* only less Sir2-9myc was bound in wild type as well as in *sas2*Δ background, respectively. (**B**) No chromatin association of Sir2-9myc above background levels (no tag) could be observed at the *CSF1* gene in wild type as well as in *sas2*Δ background. Error bars represent SD of three biological replicates.

As expected, at the telomere of the right arm of chromosome VI, Sir2-9myc was highly enriched in wild type cells, whereas there was less Sir2-9myc bound to this locus in *sas2*Δ background (Figure 28A, locus "Tel VI-R 0.5 kb"). Unexpectedly, only very little Sir2-9myc was found associated with the telomere-distal loci investigated here (Figure 28A, loci "Tel VI-R 5 kb" and "YFR054C"). Especially upon deletion of *SAS2*, this was unexpected because as the reduction of H4 K16Ac in the ChIP (Figure 25) and ChIP-chip experiments (Figure 24) indicated, the SIR complex spreads into subtelomeric regions in *sas2*Δ cells. This low association of Sir2 with subtelomeric regions in *sas2*Δ cells could be due to the fact that the SIR complex was shown not to be localized throughout the whole heterochromatin and instead is only bound to specific DNA elements, e.g. to the telomeric X element (Thurtle and Rine 2014). Thus, the subtelomeric loci investigated here ("Tel VI-R 5 kb" and "YFR054C") could be such loci of low binding of the SIR complex. Moreover, Sir2-9myc was not found to be associated with the *CSF1* gene above background levels neither in wild type nor in *sas2*Δ background (Figure 28B), which again confirmed that the decrease of H4 K16Ac at long, poorly transcribed genes seen in *sas2*Δ cells was not due to a Sir2-mediated deacetylation, but instead was a consequence of the absence of Sas2 activity.

A deletion of *SIR2* had no effect on H4 K16Ac levels at poorly transcribed genes, which in wild type cells showed high levels of H4 K16Ac and which upon deletion of *SAS2* markedly lost H4 K16Ac. This loss of H4 K16Ac in *sas2*Δ cells was not due to a Sir2-mediated deacetylation. Additionally, Sir2 could not be found to be associated with poorly transcribed genes. Collectively, the data presented here questions but does not completely exclude a genome-wide function of Sas2-mediated H4 K16Ac as a protective mark of euchromatic regions from being spuriously bound by SIR proteins. In addition to Sas2-mediated H4 K16Ac, the limited availability of SIR proteins may also prevent spurious SIR binding to euchromatic regions.

3.3 Investigations towards the dependence of Sas2-mediated H4 K16 acetylation on histone synthesis

Sas2-mediated H4 K16 acetylation was shown above to be cell-cycle dependent: Upon activation of Sas2-td, H4 K16Ac increased only in cells that passaged through S-phase, whereas in cells arrested in G1-phase, H4 K16Ac levels remained low (Figure 5B). We interpret this result to indicate that Sas2-mediated H4 K16 acetylation requires DNA replication-dependent chromatin assembly or another event in S-phase. However, an alternative hypothesis is that Sas2-mediated H4 K16 acetylation might be coupled to the synthesis of canonical histones, which occurs predominantly in S-phase (Hereford et al. 1981), when nucleosomes are assembled on the replicated DNA.

In order to address whether Sas2-mediated H4 K16 acetylation was coupled to histone synthesis, the following strategy was pursued. To avoid an increase of H4 K16Ac levels due to S-phase-coupled effects, we sought for a technical possibility to induce H4 expression outside of S-phase in cells arrested in G1-phase. To artificially induce the expression of histone H4, a galactose-inducible promoter was to be introduced in front of one H4 gene copy. In *Saccharomyces cerevisiae*, all canonical histones are encoded by two gene copies, but both copies encode the identical protein. To distinguish between the artificially induced histone H4 protein and the histone H4 that is expressed from its endogenous promoter, the galactose-inducible histone H4 was to be marked with a tag in order to increase the size of the protein, thereby making it distinguishable from the untagged H4 in a Western blot. In addition to the galactose-inducible and tagged histone H4, the experiment furthermore required an inducible Sas2 version. This was to be achieved by introducing a galactose-inducible promoter in front of the *SAS2* gene.

Having a strain with one galactose-inducible and tagged version of histone H4, one unchanged histone H4 under its endogenous promoter and a galactose-inducible Sas2, the following experiment was planned. Cells grown in medium containing glucose would be arrested in G1-phase. Subsequently, the cells would be shifted to medium containing galactose while keeping them arrested in G1-phase. Due to galactose, the tagged version of H4 as well as Sas2 would then be induced in G1-phase. If Sas2-mediated H4 K16 acetylation depends on histone synthesis, then H4 K16Ac only increases at the induced tagged histone H4 and can be visualized by Western blotting. The acetylation status of the untagged H4, which is expressed from its endogenous promoter in the S-phase before, is not expected to increase.

3.3.1 Construction of a strain with one N-terminally tagged, galactose-inducible histone H4 gene copy

In a first attempt, *HHF1* (encoding histone H4) was N-terminally tagged with three HA-tags, while simultaneously replacing the *HHF1* promoter with a galactose-inducible promoter, *GALLpr*, by applying the N-terminal tagging technique (Janke et al. 2004). This construct was then introduced into a strain bearing a galactose-inducible, N-terminally-HA-tagged version of Sas2, *GALLpr-3HA-SAS2* (constructed by Franziska Heise). Additionally, in the resulting strain, *BAR1* was deleted in order to improve the ability to arrest the cells in G1-phase using the α-factor mating pheromone.

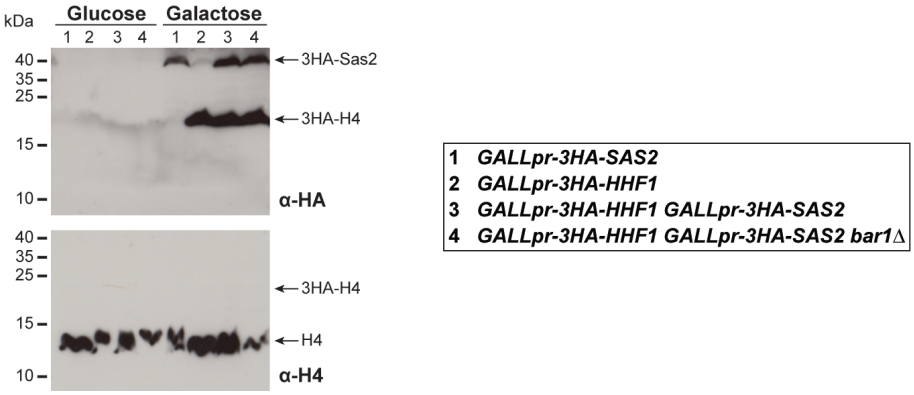

Figure 29. 3HA-tagged H4 could not be detected in α-H4 Western blot.
Strains bearing *GALLpr-3HA-SAS2* (AEY4148), *GALLpr-3HA-HHF1* (AEY5236), *GALLpr-3HA-HHF1 GALLpr-3HA-SAS2* (AEY5250) or *GALLpr-3HA-HHF1 GALLpr-3HA-SAS2 bar1*Δ (AEY5264) constructs were grown in medium containing glucose or galactose, respectively. Whole cell protein extracts of these cells were subjected to Western blotting against the HA-tag (upper blot) and against histone H4 (lower blot). Although well expressed upon induction with galactose as seen in the HA-blot, 3HA-H4 could not be detected in a Western blot against histone H4. 3HA-Sas2 is expressed upon induction in medium containing galactose.

However, although the 3HA-H4 protein was expressed upon induction in medium containing galactose and detectable with an α-HA antibody (Figure 29, upper blot), this 3HA-H4 could not be detected in a Western blot neither with an α-H4 (Figure 29, lower blot), nor with a α-H4 K16Ac antibody (data not shown). Antibodies against unmodified histone H4 and H4 K16Ac from different sources were tested, but none of them could detect 3HA-H4. We hypothesized that the N-terminal tag of 3HA-H4 masked the epitope of the antibodies tested so far, thereby preventing the binding of the tested antibodies. We therefore sought to generate a C-terminally tagged, galactose-inducible H4 protein that could potentially be detected in Western blots against H4 and H4 K16Ac.

3.3.2 Construction of a strain with one C-terminally tagged, galactose-inducible histone H4 gene copy

In a second attempt, *HHF2* (encoding histone H4) was C-terminally tagged with six HA-tags by applying the C-terminal tagging technique (Janke et al. 2004). Having successfully integrated these tags at *HHF2*, the *HHF2* promoter was replaced by a galactose-inducible promoter, *GALLpr*, by applying the N-terminal tagging technique (Janke et al. 2004). Transformants of the latter strain construction step were then tested for the detectability of the H4-6HA protein in a Western blot against histone H4.

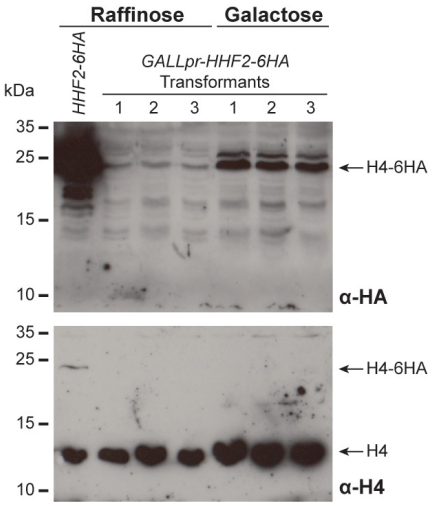

Figure 30. H4-6HA could only be detected in α-H4 Western blot when it was expressed under its endogenous promoter and not under the control of the *GALL*-promoter.
A strain bearing *HHF2-6HA* under the control of its endogenous promoter (AEY5366) as well as transformants expressing *HHF2-6HA* under the control of the *GALL*-promoter were grown in medium containing raffinose. In the transformants, *HHF2-6HA* expression was induced by adding 2% galactose to the raffinose-containing medium for 2 hours. Whole cell protein extracts of these cells were subjected to Western blotting against the HA-tag (upper blot) and against histone H4 (lower blot). *HHF2-6HA* was strongly expressed under the control of its endogenous promoter, whereas *HHF2-6HA* was moderately induced under the control of the *GALL*-promoter

in galactose-containing medium. Only when strongly expressed, as it is under the control of the endogenous promoter, H4-6HA could be detected in a Western blot against histone H4.

Under the control of its endogenous promoter, *HHF2-6HA* was strongly expressed, whereas under the control of the *GALL*-promoter, *HHF2-6HA* was moderately expressed upon induction with galactose (Figure 30, upper blot). Thus, surprisingly, the endogenous promoter of *HHF2* was substantially stronger than the induced *GALL*-promoter. Consequently, H4-6HA was only detectable when expressed in high amounts under the control of the endogenous *HHF2*-promoter in a Western blot with an α-H4 antibody (Figure 30, lower blot). Since the *GALL*-promoter apparently was not strong enough to drive the expression of H4-6HA such that it was detectable in a Western blot against histone H4, in a next step, the *HHF2* promoter was replaced with the stronger galactose-inducible *GAL1*-promoter by applying the N-terminal tagging technique (Janke et al. 2004). Transformants of this strain construction step were then tested for the detectability of the H4-6HA protein in Western blots against histone H4 and H4 K16Ac, respectively.

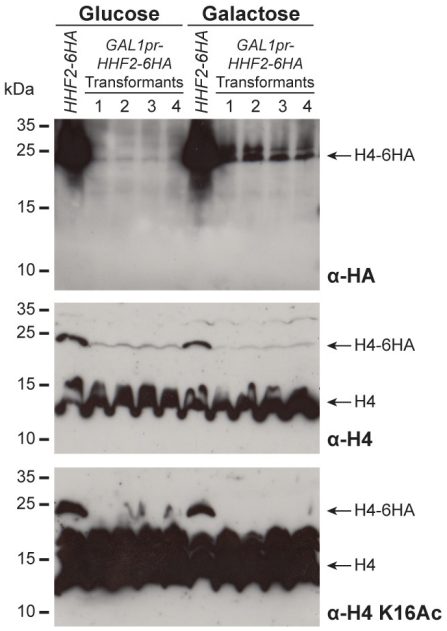

Figure 31. H4-6HA could not be detected in α-H4 and α-H4 K16Ac Western blots when it was expressed under the control of the *GAL1*-promoter.
A strain bearing *HHF2-6HA* under the control of its endogenous promoter (AEY5366) as well as transformants expressing *HHF2-6HA* under the control of the *GAL1*-promoter were grown in medium containing 2% raffinose. 2% glucose or galactose was added to the medium and cells were grown for two hours, respectively. Whole cell protein extracts of these cells were subjected to Western blotting against the HA-tag (upper blot), against histone H4 (middle blot) and against H4 K16Ac (lower blot). *HHF2-6HA* was strongly expressed under the control of its endogenous promoter in glucose- as well as galactose-containing medium, whereas *HHF2-6HA* was only moderately induced under the control of the *GAL1*-promoter in galactose-containing medium. Only when strongly expressed, as it was under the control of the endogenous promoter, H4-6HA could be detected in Western blots against histone H4 and H4 K16Ac.

Again, under the control of its endogenous promoter, *HHF2-6HA* was strongly expressed in glucose- as well as galactose-containing medium, whereas under the control of the *GAL1*-promoter, *HHF2-6HA* was only moderately expressed upon induction with galactose (Figure 31, upper blot). Thus, the endogenous promoter of *HHF2* was also substantially stronger than the *GAL1*-promoter. As before, H4-6HA only was detectable in a Western Blot against histone H4 and H4 K16Ac when expressed in high amounts under the control of the endogenous *HHF2*-promoter (Figure 31, middle and lower blot).

In order to enrich for H4-6HA upon induction with galactose, in a next step, we attempted to immunoprecipitate H4-6HA using an antibody against histone H4. An immunoprecipitation using an antibody directed against the HA-tag would not be helpful here, because the untagged histone H4, which was expressed under its endogenous promoter in S-phase, would thereby be excluded from the analysis, although it is essential for the experiment in order to compare H4 K16Ac levels of the tagged and the untagged histone H4 upon induction of *SAS2*. The immunoprecipitation was performed as described (Nourani et al. 2001), using lysates of cells expressing *HHF2-6HA* under the control of the *GAL1*-promoter and as control in lysates of cells expressing *HHF2-6HA* under the control of the endogenous *HHF2*-promoter.

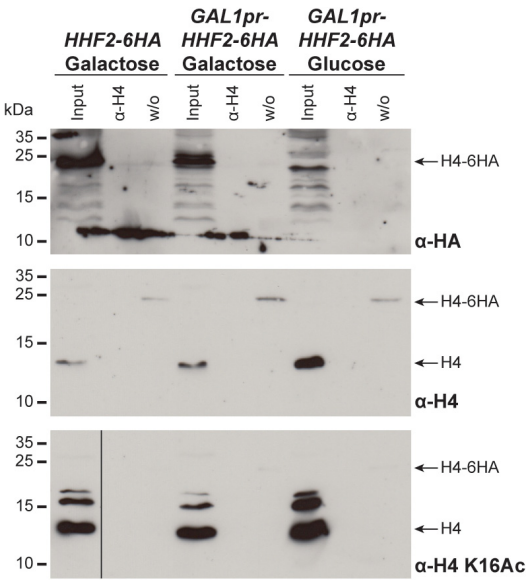

Figure 32. H4-6HA could not be enriched by immunoprecipitation with an antibody against histone H4. Cells expressing *HHF2-6HA* under the control of the endogenous promoter (AEY5366) as well as cells expressing *HHF2-6HA* under the control of the *GAL1*-promoter (AEY5403) under inducing (galactose) or inhibiting conditions (glucose) were subjected to immunoprecipitation against histone H4 to enrich for H4-6HA. The α-H4 antibody was crosslinked to the Protein G agarose beads that were used. Input lysats as well as immunoprecipitation samples treated with and without (w/o) the α-H4 antibody were analyzed by Western blotting against the HA-tag (upper blot), histone H4 (middle blot) and H4 K16Ac (lower blot). H4-6HA could not be immunoprecipitated with the used α-H4 antibody as seen by the lack of a band in the α-H4 samples of lysats of *HHF2-6HA* as well as *GAL1pr-HHF2-6HA* in galactose-containing medium. Thus, also no signal in Western blots against histone H4 and H4 K16Ac could be detected.

Neither under the control of the endogenous *HHF2*-promoter, nor under the control of the *GAL1*-promoter under inducing conditions, could H4-6HA be immunoprecipitated using an antibody against unmodified histone H4 (Figure 32, upper blot). Therefore, it was not surprising that H4-6HA could not be detected in Western blots against histone H4 and H4 K16Ac (Figure 32, middle and lower blot). The 6HA-tag did not perturb the binding of the α-H4 antibody used for the immunoprecipitation, because untagged histone H4 also was not immunoprecipitated, as it could not be detected in the α-H4 samples in Western blots against histone H4 and H4 K16Ac (Figure 32, middle and lower blot).

So far, the *GAL1pr-HHF2-6HA* construct was genomically integrated, i.e. one copy of the construct was present per haploid cell. As shown above (Figure 31), when expressed in high amounts under the control of the endogenous *HHF2*-promotor, H4-6HA could be detected in Western blots against H4 and H4 K16Ac, respectively. Thus, in a final attempt, the *GAL1pr-HHF2-6HA* construct was introduced onto a yeast 2μ plasmid (pAE2105) and transformed into yeast cells to increase the number of copies of the construct per cell (one cell can carry 50-100 copies of the 2μ plasmid). The expression of H4-6HA in *GALLpr-3HA-SAS2* cells carrying the *GAL1pr-HHF2-6HA* construct on a 2μ plasmid was then addressed by Western blotting against the HA-tag, histone H4 and H4 K16Ac under inducing (galactose) and repressing (glucose) conditions.

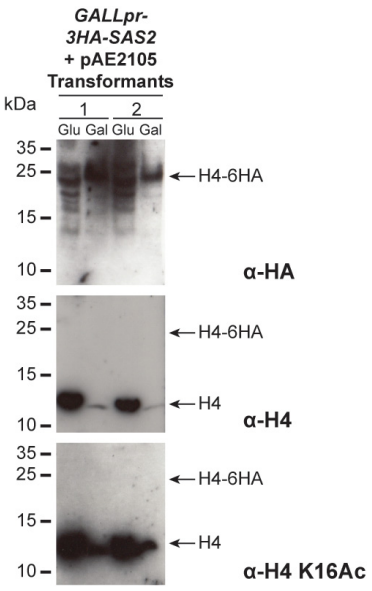

Figure 33. H4-6HA could not be detected in α-H4 and α-H4 K16Ac Western blots when it was expressed from a 2μ plasmid under the control of the *GAL1*-promoter.
Yeast cells bearing *GALLpr-3HA-SAS2* (AEY4148) were transformed with a 2μ plasmid carrying the *GAL1pr-HHF2-6HA* construct (pAE2105). By Western blotting against the HA-tag (upper blot), histone H4 (middle blot) and H4 K16Ac (lower blot), respectively, the expression of H4-6HA under inducing (Gal, galactose for 2 h) and inhibiting conditions (Glu, glucose for 2 h) was tested in two transformants. In medium containing galactose, H4-6HA was well expressed (upper blot), but this H4-6HA could not be detected in Western blots against

histone H4 (middle blot) and H4 K16Ac (lower blot). Upon induction of H4-6HA expression, levels of untagged H4 were reduced in both transformants, as seen in the Western blots against H4 as well as H4 K16Ac.

Upon expression of H4-6HA from a 2µ plasmid, H4-6HA could readily be detected in a Western blot against the HA-tag (Figure 33, upper blot). However, H4-6HA again could not be detected in Western blots neither against histone H4 (Figure 33, middle blot), nor against H4 K16Ac (Figure 33, lower blot). Thus, even when the number of *GAL1pr-HHF2-6HA* copies was markedly increased, as was the case for the expression from a 2µ plasmid, the amount of H4-6HA was not sufficient to be detected in Western blots against histone H4 and H4 K16Ac. Surprisingly, when H4-6HA expression was induced, the amount of untagged histone H4, which was expressed under the control of the endogenous promoter, was reduced (Figure 33, middle and lower blot). This effect was observed repeatedly and suggested that strong induction of H4-6HA resulted in a compensatory downregulation of the endogenous gene copy.

Collectively, a strain expressing a C-terminally 6HA-tagged version of histone H4 under the control of the galactose-inducible *GAL1*-promoter could be generated. However, this H4-6HA protein could only be detected in Western blots against the HA-tag, but not in Western blots against histone H4 or H4 K16Ac, which was a prerequisite for the experiment this strain was planned to be used for (3.3). Even when expressed from a 2µ plasmid with a high copy number per cell, H4-6HA was not detectable in Western blots against histone H4 or H4 K16Ac. Moreover, this expression from a 2µ plasmid also caused the downregulation of the expression of the untagged histone H4 copy, whose expression was limited to S-phase. Due to the lacking detectability of H4-6HA, the experiment addressing the dependence of Sas2-mediated H4 K16 acetylation on histone synthesis unfortunately could not be performed.

4 Discussion

In *Saccharomyces cerevisiae*, the SAS-I complex with its catalytic subunit, the MYST histone acetyltransferase Sas2, prevents the spreading of SIR-mediated heterochromatin at telomeres (Kimura et al. 2002; Suka et al. 2002) and is involved in silencing of the silent mating-type loci (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997) and the rDNA locus (Meijsing and Ehrenhofer-Murray 2001). Moreover, Sas2-mediated acetylation of H4 K16 has a genome-wide function in that it negatively affects the rate of transcription elongation (Heise 2011; Heise et al. 2012). In this study, we investigated when H4 K16Ac is deposited into chromatin and which chromatin assembly factors / histone chaperones influence the deposition. We found that bulk H4 K16 was acetylated by Sas2 during the S-phase of the cell cycle. However, this H4 K16Ac was not incorporated into chromatin during the same S-phase. Furthermore, we could show that Sas2-mediated H4 K16 acetylation during S-phase depended on the function of the chromatin assembly factors Asf1 and CAF-1, which both interact with Sas2 (Meijsing and Ehrenhofer-Murray 2001). Furthermore, we found that H4 K16Ac became enriched at ORFs during gene repression, e.g. at the *GAL* genes, in a Sas2-dependent manner. This enrichment was not seen in cells in which the histone chaperone Spt6 was not functional. In these *spt6-1004* cells, histone H4 occupancy at ORFs was low, while relative H4 K16Ac levels were high compared to wild type cells. This suggested an indirect effect of Spt6 on H4 K16Ac levels in that Spt6 incorporates K16-unacetylated histone H4 and thereby dilutes H4 K16Ac. Furthermore, we could show that H4 K16Ac levels in euchromatic regions were not influenced by the HDAC Sir2, which argued that Sas2-mediated H4 K16Ac prevented spurious binding of the SIR complex at subtelomeric genes, but not in other euchromatic regions.

4.1 Sas2 acetylated H4 K16 during the S-phase of the cell cycle

Taking advantage of a Sas2 degron strain, with which Sas2 could efficiently be switched on and off, we could show that Sas2 acetylated H4 K16 in the S-phase of the cell cycle, but not in G1-phase. The genes encoding histone H4 (*HHF1* and *HHF2*) are expressed during S-phase to counter the need for histones to package the duplicated genome (Hereford et al. 1981). Thus, one interpretation of the S-phase-dependence of Sas2-mediated H4 K16 acetylation is that Sas2 has specificity for newly synthesized histones. However, expression of canonical histones is not completely absent during G1-phase (Verzijlbergen et al. 2010), which would allow for an increase of H4 K16Ac in G1-phase. Yet, we did not observe an

increase of H4 K16Ac during G1-phase, which suggested that Sas2 does not have specificity for newly synthesized histone H4. Alternatively, the SAS-I complex might by activated by a cell cycle-dependent event that is restricted to S-phase, possibly a posttranslational modification, which so far has not been shown and needs to be investigated further.

The S-phase-dependence of Sas2-mediated H4 K16 acetylation was further supported by the observation that the increase of H4 K16Ac during S-phase was reduced upon deletion of *ASF1* and *CAC1*, respectively. The chromatin assembly factor 1 (CAF-1) with its subunit Cac1 almost exclusively assembles nucleosomes on replicated DNA during S-phase (Smith and Stillman 1989). The histone chaperone Asf1 is involved in replication-coupled nucleosome assembly (Tyler et al. 1999), but is also active outside of S-phase. Both CAF-1 and Asf1 interact with Sas2 (Meijsing and Ehrenhofer-Murray 2001). Thus, it seems likely that, during S-phase, Asf1 and CAF-1 bind and present histone H4 to the SAS-I complex for acetylation. This is consistent with the fact that histone H4, which is associated with CAF-1, was shown to be acetylated on H4 K16 (Zhou et al. 2006). However, association of H3 and H4 with Asf1 blocks the acetylation of H4 K16 by the SAS-I complex (Sutton et al. 2003). Thus, perhaps Asf1 passes K16-unacetylated H4 to CAF-1, which then presents it for acetylation to SAS-I and deposits H4 K16Ac onto the replicated DNA. However, CAF-1 and Asf1 seemingly contribute to the rate of H4 K16 acetylation, but not to the steady-state level, since, in *asf1*Δ and *cac1*Δ cells, H4 K16Ac eventually becomes incorporated into chromatin, leading to only mild changes in genome-wide H4 K16Ac levels compared to wild type cells (Heise 2011).

Surprisingly, although H4 K16 was acetylated during S-phase upon Sas2-td activation, H4 K16Ac was not incorporated into chromatin in a genome-wide fashion. Instead, it only became enriched at genes that became repressed in the course of the experiment (*GAL* genes and a heat shock gene) in S-phase, but also in G1-phase. Of note, expression of canonical histones starts in late G1-phase immediately prior to initiation of DNA replication (Hereford et al. 1981), and H4 K16Ac levels increase early in S-phase, reaching the highest level of H4 K16Ac already after 20 min of release from a G1-arrest (Wilkins et al. 2014). Hence, one explanation for the absence of chromatin incorporation of H4 K16Ac could be that H4 K16 has to be acetylated before nucleosome reassembly in the wake of the DNA replication machinery initiates. Thus, in the cell-cycle experiment using the *SAS2-td* strain, H4 K16Ac perhaps was provided too late to be deposited into chromatin during the same S-phase, since Sas2-td in this experiment was activated as cells were released into S-phase. Alternatively, it is possible that H4 K16Ac first appears in a cellular pool bound to chromatin assembly

factors, which serves as a reservoir for H4 to be deposited in a later step or in the next S-phase, which was not investigated in the experiments conducted in this study.

Moreover, the lack of chromatin incorporation in bulk H4 K16Ac also suggested that the SAS-I complex does not acetylate histone H4 that is associated with DNA. Instead, it is most likely that SAS-I acetylates free or to some extent CAF-1-bound histone H4, further implying the existence of a nuclear pool of H4 K16Ac. Again, it has been shown that association of H3 and H4 with Asf1 blocks H4 K16 acetylation by the SAS-I complex (Sutton et al. 2003). The Sas2-mediated acetylation of free histone H4 is in agreement with the fact that, by ChIP, Sas2 is not found associated with chromatin except at the rDNA locus (Meijsing and Ehrenhofer-Murray 2001; Heise 2011). Alternatively, chromatin association of Sas2 might be transient, for instance during S-phase, or not amenable to ChIP due to technical reasons. However, the in vitro activity of SAS-I is stronger on free histone H4 than on nucleosomes (Sutton et al. 2003), and thus, Sas2-mediated acetylation of chromatin-bound histone H4 is unlikely.

4.2 Sas2-mediated H4 K16Ac became enriched upon gene repression and was indirectly influenced by Spt6

Upon acetylation during S-phase by Sas2, H4 K16Ac was incorporated into chromatin at ORFs not only in S-phase, but also in G1-phase, upon repression of a gene. This observation of high levels of H4 K16Ac in correlation with low levels of gene expression is in agreement with earlier studies in *S. cerevisiae* (Kurdistani et al. 2004; Liu et al. 2005; Heise et al. 2012). Of note, a deletion of *SAS2*, which leads to a reduction of H4 K16Ac at the majority of ORFs, was found to cause a mildly elevated RNA Pol II occupancy at a subset of genes (Heise 2011; Heise et al. 2012), which suggested that H4 K16Ac and the presence of Sas2 is slightly inhibitory to transcription. Thus, the observation that H4 K16Ac became enriched upon gene repression was in agreement with high levels of H4 K16Ac correlating with low transcription levels. However, this correlation is still unanticipated because H4 K16Ac was reported to be associated with open chromatin, since it inhibits the formation of higher order chromatin structures (Shogren-Knaak et al. 2006). Furthermore, transcription elongation has been linked to histone deacetylation mediated by the RNA Pol II dependent recruitment of the HDAC Rpd3S, which is important to restore a repressive chromatin structure after transcription (Carrozza et al. 2005; Joshi and Struhl 2005; Keogh et al. 2005). Thus, in *S. cerevisiae*, H4 K16Ac is not a repressive mark per se, as e.g. methylated H3 K9 in higher eukaryotes (Nakayama et al. 2001). Instead, it apparently is incorporated into chromatin to serve a different function, which will be discussed later (4.3).

In addition to Rpd3-containing complexes (Carrozza et al. 2005; Keogh et al. 2005; Li et al. 2007b), also other HDACs, for instance Hda1 and Hos2 (Govind et al. 2010), are recruited to the ORFs during transcription. Therefore, perhaps the dissociation of these HDACs from the ORFs upon gene repression may also contribute to the increase of H4 K16Ac that was observed. However, if this was the predominant mechanism of H4 K16Ac increase upon gene repression, we would also expect such an increase in *sas2*Δ cells. However, the observed enrichment of H4 K16AC was strictly Sas2-dependent, arguing that the H4 K16Ac increase was not solely due to a reduced activity of HDACs at the repressed ORF.

The increase of H4 K16Ac during transcriptional repression was influenced by the histone chaperone Spt6 in an indirect manner. When Spt6 function was impaired, relative H4 K16Ac levels at genes were high independently of the transcriptional status of the gene, whereas H4 occupancy was low at these genes. Our data, together with earlier work by others (Ivanovska et al. 2011; Perales et al. 2013), suggest the following model. As the transcription machinery moves through the coding region of a gene, histone H4 acetylated or unacetylated at K16 together with other histones partially or completely dissociates from the DNA. Whether H4 along with H3 is evicted by histone chaperones or whether it is displaced by the progressing RNA polymerase is not known in detail. In order to reassemble nucleosomes in the wake of RNA polymerase, "old" histone H4 (K16-acetylated), but also new, K16-unacetylated histone H4 are deposited. Via its interaction with the progressing RNA Pol II (Yoh et al. 2007), the histone chaperone Spt6 is recruited to the coding region, where it incorporates new, K16-unacetylated H4 into chromatin behind the RNA polymerase. When the transcription level is low or a gene is repressed, less RNA Pol II and thus less Spt6 moves along the gene body. Therefore, the reincorporation of K16-acetylated histone H4 predominates over the incorporation of K16-unacetylated H4, thereby leading to increased levels of H4 K16Ac over the ORF. Thus, if the transcription rate is low, more H4 K16Ac than K16-unacetylated H4 is incorporated in the wake of RNA Pol II.

There are two possibilities for how H4 K16Ac could be introduced or maintained at poorly expressed or repressed genes. On the one hand, "old", K16-acetylated H4 may remain associated with the DNA after H2A/H2B-dimer eviction by FACT. This "old" H4, together with "old" H3 may then be "passed back" from in front to behind the elongating RNA Pol II by the transcription machinery itself (Studitsky et al. 1994; Studitsky et al. 1997). Due to the fact that this "pass back" mechanism may decelerate transcription in comparison to transcription on a completely "naked" DNA template, this mechanism is expected to be more pronounced in genes with a low expression rate, as has been observed (Radman-Livaja et al.

2011). On the other hand, "old" H4 K16Ac together with H3 might be evicted from the DNA ahead and redeposited in the wake of the progressing RNA Pol II. The eviction could be mediated by RNA Pol II itself, but this is only observed during strong transcription (Kristjuhan and Svejstrup 2004; Schwabish and Struhl 2004). Alternatively, "old" H4 K16Ac could be evicted by an unknown factor, which might also mediate the local redeposition behind the RNA Pol II. Spt6 is not likely to be involved in the eviction of H4 K16Ac, because it is known as a histone deposition rather than a disassembly factor (Bortvin and Winston 1996; Kaplan et al. 2003), and thus would have to display a different binding specificity towards H4 K16Ac during the eviction of histones ahead of RNA Pol II. The histone chaperone Asf1 travels with elongating RNA Pol II and is involved in the eviction and redeposition of histones ahead and in the wake of RNA Pol II, respectively (Schwabish and Struhl 2006). In this context, Asf1 might serve as an acceptor for H4 K16Ac during a first round of transcription upon transcriptional activation of a gene. Thus, Asf1 might be a regulator of the nuclear H4 K16Ac pool, as already suggested by the Asf1-dependent H4 K16 acetylation during S-phase. However, we did not observe a significant effect on the enrichment of H4 K16Ac during transcriptional repression of *GAL10* in *asf1*Δ cells.

Taken together, we hypothesize that H4 K16Ac is incorporated into chromatin on a genome-wide scale during S-phase. In euchromatic regions, H4 K16Ac patterns are then modulated by dynamic processes requiring access to the DNA, e.g. transcription. At highly expressed genes, H4 K16Ac is almost completely replaced by K16-unacetylated H4, which is mediated by Spt6. At poorly expressed genes, H4 K16Ac is likely to be maintained by a "pass back" mechanism possibly mediated by RNA Pol II. Upon repression of a highly expressed gene, H4 K16Ac most likely originating from a nuclear pool of chaperone bound H4 K16Ac becomes enriched. The observation that a deletion or functional disruption of one histone chaperone alone did not influence the incorporation of H4 K16Ac during repression suggests that the pool of H4 K16Ac is collectively and redundantly maintained by different histone chaperones, most likely by Asf1, CAF-1 and Rtt106, since Asf1 and CAF-1 interact with Sas2 (Meijsing and Ehrenhofer-Murray 2001), and Rtt106 is known to be active during S-phase (Huang et al. 2005), when H4 K16 is acetylated by Sas2.

4.3 What is the genome-wide function of Sas2-mediated H4 K16Ac?

At telomeres in *S. cerevisiae*, Sas2-mediated H4 K16Ac has a boundary function in preventing the spreading of SIR-characterized heterochromatin into adjacent euchromatic regions (Kimura et al. 2002; Suka et al. 2002). Upon deletion of *SAS2*, this spreading of the

SIR complex into subtelomeric euchromatic regions leads to gene repression, which, if SIR spreading is excessive (Ehrentraut et al. 2010), can affect cell viability. Anticipating SIR-mediated gene repression is of particular importance at genes with a finely balanced, low expression level, which correlates with a low histone turnover, because silencing of such genes could potentially have severe effects on cell proliferation. Consequently, such poorly expressed genes have high levels of H4 K16Ac (Heise et al. 2012). Thus, it is most likely that H4 K16Ac is incorporated during S-phase on a genome-wide scale to protect genes from being spuriously bound by SIR proteins. This is supported by the observation that, upon deletion of *SAS2*, H4 K16Ac levels are decreased on a genome-wide scale compared to wild type cells, and this decrease is specifically pronounced at coding-regions, whereas intergenic regions were largely unaffected (Heise et al. 2012). Moreover, H4 K16Ac levels are strongly reduced at long, poorly transcribed genes in *sas2*Δ cells. In this study, we found that this decrease in euchromatic genes was due to the absence of Sas2-mediated H4 K16 acetylation activity, and not due to erroneous euchromatic binding of SIR/Sir2, which could potentially deacetylate H4 K16 at these euchromatic genes. Thus, this observation argued for a predominant function of Sas2-mediated H4 K16Ac in preventing binding of the SIR complex at subtelomeric, but not euchromatic genes. Furthermore, this illustrates that SIR propagation into euchromatic regions may primarily be limited by the available amount of SIR proteins (Kueng et al. 2013), because upon reduction of H4 K16Ac levels in *sas2*Δ cells, Sir2 was not associated with euchromatic genes that normally show high levels of H4 K16Ac in wild type cells. Thus, as others (Kimura et al. 2002; Suka et al. 2002; Ehrentraut et al. 2010) and we could show, by counteracting SIR-mediated silencing, H4 K16Ac maintains genes amenable to transcription only at subtelomeric euchromatic regions, and not on a genome-wide scale. However, the Sas2-mediated H4 K16 acetylation activity at telomeres is identical to that throughout the genome, since a deletion of *SAS2* had similar effects on H4 K16Ac levels at telomeres in *sir2*Δ cells as in any euchromatic region in wild type cells.

Assuming that H4 K16Ac is incorporated into chromatin during S-phase on a genome-wide scale, H4 K16Ac levels are then modulated due to different chromatin environments. At telomeres, for instance, H4 K16Ac is deacteylated by the SIR component Sir2 (Imai et al. 2000) during the establishment and spreading of SIR-mediated heterochromatin. Low levels of H4 K16Ac are furthermore established at highly expressed genes by the activity of the transcription elongation factor Spt6, which incorporates K16-unacetylated H4 instead of H4 K16Ac in the wake of the progressing RNA Pol II. At poorly transcribed genes, high levels of H4 K16Ac are maintained by a nucleosome pass back mechanism mediated by RNA

Pol II or by nucleosome dis- and reassembly mediated by histone chaperones. Thus, differences in H4 K16Ac levels in different genomic regions may not be the consequence of active, differential incorporation of H4 K16Ac, but instead are the consequence of different processes acting on chromatin, thereby changing H4 K16Ac levels, e.g. transcription, nucleosome remodeling, heterochromatin spreading. This causal mode to establish different patterns of chromatin marks has been postulated by Henikoff and Shilatifard (2011).

As was observed in this study, the only case, in which H4 K16Ac is actively incorporated into chromatin outside of S-phase, is during transcriptional repression of a highly expressed gene, e.g. *GAL* genes. In this case, H4 K16Ac might be provided from a nuclear pool of histone H4 that is bound by histone chaperones. As we could show, H4 K16Ac is not needed to prevent SIR-mediated silencing at euchromatic genes. However, the active incorporation of H4 K16Ac upon gene repression outside of S-phase suggests a functional relevance for Sas2-mediated H4 K16Ac in euchromatin. Which function this is, remains to be investigated further.

In *S. cerevisiae*, H4 K16Ac is associated with low transcription rates, but it is not the cause for transcriptional repression, and furthermore, it prevents SIR-mediated silencing at specific loci, but not on a genome-wide scale. Thus, H4 K16Ac is incorporated to maintain genes amenable to transcription. This function for H4 K16Ac is unanticipated, since in all other higher eukaryotes investigated so far, H4 K16Ac is associated with transcriptional activity. For example in *Drosophila melanogaster*, H4 K16Ac mediated by the MSL complex is involved in the two-fold upregulation of the expression of X-chromosome-encoded genes in male flies, which is necessary for dosage compensation (Akhtar and Becker 2000; Smith et al. 2000; Kind et al. 2008). Additionally, H4 K16Ac mediated by the NSL complex also regulates the constitutive expression of housekeeping genes in *D. melanogaster* (Lam et al. 2012). Moreover, in mammalian cells, a loss of hMOF-mediated H4 K16Ac is associated with transcriptional repression (Smith et al. 2005; Gupta et al. 2008; Li et al. 2009). Thus, compared to higher eukaryotes, H4 K16Ac in *S. cerevisiae* has a different functional significance: preventing SIR-mediated silencing rather than keeping chromatin open to support transcriptional activity.

4.4 H4 K16Ac during the cell cycle

During the S-phase of the cell cycle, DNA together with chromatin has to be faithfully duplicated. As has been proposed before (Meijsing and Ehrenhofer-Murray 2001), in this study, we found that H4 K16 is acetylated by Sas2 during S-phase, but not during G1-phase.

Of note, we did not observe an S-phase-specific incorporation of H4 K16Ac into chromatin, which was most likely due to the experimental design. During S-phase, parental H4 K16Ac is diluted due to genome duplication, which would provide the risk of inappropriate gene repression by inappropriate SIR spreading. Thus, it is reasonable to assume that Sas2-mediated H4 K16Ac is incorporated into chromatin during S-phase on a genome-wide scale. This is also supported by the fact that Sas2 interacts with the chromatin assembly factors Asf1 and CAF-1 (Meijsing and Ehrenhofer-Murray 2001), which both assemble H3/H4 on replicated DNA (Smith and Stillman 1989; Kaufman et al. 1997; Tyler et al. 1999). After its incorporation, Sas2-mediated H4 K16Ac is sculpted by different processes, e.g. transcription, keeping H4 K16Ac levels high in regions of low histone turnover to prevent SIR-mediated silencing, especially at subtelomeric regions, as discussed above (4.3).

However, the observation by Wilkins et al. (2014) that H4 K16Ac levels drop on a genome-wide scale during G2/M-phase in order to promote chromosome condensation raises the question how SIR spreading is prevented in late M- and early G1-phase. Wilkins et al. (2014) show that Aurora B-mediated phosphorylation of H3 S10 leads to the recruitment of the HDAC Hst2 that subsequently removes the acetyl group from H4 K16, freeing the H4 tail to interact with the surface of neighboring nucleosomes, thereby promoting chromosome condensation. Moreover, hypoacetylated H4 K16 at centromeres is essential for accurate chromosome segregation during M-phase (Choy et al. 2011). Thus, during M-phase, H4 K16 is largely deacetylated on a genome-wide level to promote chromosome condensation and chromatid segregation. Assuming that during M-phase all chromatin-bound H4 K16Ac is deacetylated and new H4 K16Ac is incorporated on a genome-wide scale only in S-phase, it remains to be answered how euchromatic regions are protected from SIR-mediated silencing during G1-phase under these conditions. Presumably, a limited amount of H4 K16Ac is stored in a histone chaperone-bound pool, which serves as a source for H4 K16Ac to be incorporated into chromatin, e.g. during transcriptional repression, as we observed for *GAL10* during G1-phase. However, this pool is not likely to be large enough to suffice for all euchromatic regions, especially for those adjacent to heterochromatic regions and with low histone turnover being most vulnerable to SIR-mediated silencing. Alternatively, the limited amount of available SIR proteins could prevent its spreading into euchromatic regions. However, it is conceivable that especially at boundary regions between heterochromatin and euchromatin, SIR proteins might spread excessively. Perhaps the second HAT acetylating H4 K16 in *S. cerevisiae*, Esa1 (Smith et al. 1998), partially compensates for the loss of H4 K16Ac during M-phase, for instance at subtelomeric loci. However, it needs to be investigated how the M-

phase-associated loss in H4 K16Ac is compensated during G1-phase to prevent excessive SIR spreading.

4.5 Causes of experimental difficulties in this study

In the course of this study, several experimental difficulties were encountered: high variation of ChIP signals in biological replicates, lacking detectability of a tagged version of histone H4 expressed from a heterologous promoter and difficulties in cell cycle synchronization and S-phase release (see Appendix). Causes of these experimental difficulties will be discussed in this section.

4.5.1 The need for and the problems with biological replicates

Reproducibility is a key requirement to evaluate the results of an experiment and to draw conclusions regarding the significance of observed effects. For biological experiments, it is established practice to repeat experiments at least three times under the same conditions, in which most significant conclusions, also about the underlying variability in measurement, can be drawn from biological replicates. In the case of experiments with *S. cerevisiae*, for example, three biological replicates correspond to three separate yeast cultures to be independently worked with. However, as seen in the chromatin immunoprecipitation experiments conducted in this study, the variation between biological replicates as illustrated by standard deviation can be high. Thus, in some cases, observed effects could not be classified as statistically significant, but rather, may indicate a trend. Hence, the question remains why the variation between biological replicates is so high in some cases.

Understandably, variation may result from variations in the experimental procedure. For example in ChIP experiments, only small amounts of DNA are immunoprecipitated. By pipetting, some DNA could be lost, which could have severe impact on the measured results. This error cannot be classified as systematic, since it may be different in different samples. However, by carefully performing the experiments, the experimantator can try to reduce such variations in the experimental procedure to a minimum. Assuming that the experimental procedure, i.e. culturing of the cells and the ChIP procedure, was the same in all biological replicates, then the measured variation may represent the biological variation, i.e. the variation between different cultures of the same yeast strain. Biological variation is the result of a broad spectrum of factors, which will be discussed exemplarily in a qualitative manner and not with respect to statistics.

One cause of biological variation between replicates may be environmental factors. As we observed, for instance cell cycle synchrony can vary between replicates and the cellular reaction to changes in the culturing conditions, e.g. during a shift from galactose- to glucose-containing medium, can be different in different replicates, although the experimental procedure for all replicates was the same. However, the influence of environmental factors, which could cause biological variation, remains speculative, since these factors should almost be excluded due to a carefully and reproducibly performed experimental procedure.

Furthermore, genotypic variation may contribute to biological variation. Very recently, it was shown that mutation of any single gene could cause a genomic imbalance with consequences sufficient to drive adaptive genetic changes. Teng et al. (2013) analyzed a yeast knockout collection and found that most gene knockout strains had one additional mutant gene affecting nutrient responses and/or heat-stress-induced cell death. Even different colonies of the same strain from the same culturing plate displayed different growth phenotypes in response to different stresses due to variation in their genetic background. This genetic variation between equally treated colonies of the same yeast strain may contribute to the biological variation observed in the ChIP experiments in this study. Since the probability to acquire and maintain a mutation increases with age, effects of genetic variation, which result in biological variation, may also be enhanced by increasing age of a cell. Thus, if cells of the same strain but of different ages were compared with each other (which cannot be excluded), this may be another cause of the observed biological variation.

However, how the described causes for biological variation may influence for example relative H4 K16Ac levels at certain genes remains to be investigated and illustrates how many factors, genetic as well as environmental or epigenetic, may cooperate to establish and maintain a specific phenotype. Thus, for some experiments, it may also be applicable to contemplate and evaluate experimental results individually for each biological replicate to avoid a loss of experimental evidence due to averaging of the results of biological replicates.

4.5.2 Increasing the expression of one gene copy of histone H4 decreased the expression from the second gene copy

In order to investigate a potential dependence of Sas2-mediated H4 K16 acetylation on newly synthesized histone H4, a yeast strain was generated, in which one of the two gene copies encoding histone H4 (*HHF1* and *HHF2*) could be induced outside of S-phase, in which canonical histones are normally expressed (Hereford et al. 1981). By the addition of an epitope-tag, the inducible H4 could be distinguished from the untagged H4, which was

expressed under its endogenous promoter. Unfortunately, the experimental approach this strain was generated for could not be conducted, because the epitope-tagged version of histone H4 could not be detected by Western blotting against H4 K16Ac or unmodified histone H4. Nevertheless, a surprising phenomenon was observed: Upon induction of the expression of the tagged histone H4, the expression of the untagged H4, which was under its endogenous promoter, was downregulated. This confirmed the already observed dosage compensation of yeast histone genes (Osley and Hereford 1981). Although the transcriptional rate of H4-6HA was increased due to the galactose-mediated induction, the steady-state levels of histone H4 mRNAs (tagged as well as untagged) may be constant due to increased posttranscriptional mRNA turnover. This demonstrated the need to prevent the enrichment of excessive amounts of histone proteins, especially outside of S-phase, which would otherwise unspecifically bind to DNA and eventually cause cell death.

4.5.3 Difficulties in cell cycle synchronization and S-phase release

The HAT Sas2 interacts with the chromatin assembly factors Asf1 and CAF-1 (Meijsing and Ehrenhofer-Murray 2001), which deposit H3/H4 onto replicated DNA as a first step in nucleosome assembly during S-phase (Smith and Stillman 1989; Kaufman et al. 1997; Tyler et al. 1999). In addition, both these chromatin assembly factors interact with the sliding clamp PCNA (Huang et al. 2005). Single deletions of *ASF1* or subunits of CAF-1 (*CAC1*, *CAC2*, *CAC3*) as well as double deletions of *ASF1* in combination with a subunit of CAF-1 are not lethal in *S. cerevisiae*, but show effects on silencing at telomeres and silent mating-type loci (Tyler et al. 1999). This has led to the hypothesis, that CAF-1 as well as Asf1 predominantly assemble nucleosomes at heterochromatic loci within the yeast genome (Tyler 2002). During the course of this study, a genome-wide map of chromatin association of CAF-1 during a whole S-phase should be generated using a ChIP-sequencing approach (see Appendix). However, due to problems in cell-cycle synchronization, this experiment could not be conducted.

Cells could be efficiently arrested in G1-phase by the addition of the α-factor mating pheromone regardless of whether *BAR1*, which encodes the protease that cleaves α-factor, was deleted or not. However, when the cells were released into S-phase, a subpopulation of cells remained in G1-phase and entered S-phase belatedly. This asynchrony in the S-phase release concomitantly caused an asynchrony in DNA replication in the whole culture, which led to a lack in traceability of the replication machinery at distinct loci due to overlaying signals from different subpopulations of cells. This asynchronous S-phase release could not

be overcome by any changes in the experimental design. The reason for this cell-cycle asynchrony remains enigmatic, since this synchronization approach is well established and is used in many labs (Aparicio et al. 1997; Vogelauer et al. 2002; Azvolinsky et al. 2009).

4.6 Summary and outlook

In this study, it was shown that H4 K16 was acetylated by Sas2 during S-phase in an Asf1- as well as CAF-1-dependent manner. Likely, Sas2-mediated H4 K16Ac is also incorporated into chromatin on a genome-wide scale during S-phase, which, nevertheless, needs to be investigated using the Sas2 degron strain, but a different experimental design for cell cycle synchronization and activation of Sas2-td. Furthermore, it needs to be determined how Sas2 activity is restricted to S-phase, which is maybe achieved by cell cycle-dependent posttranslational modifications of the SAS-I complex. Upon repression of *GAL10*, Sas2-mediated H4 K16Ac was incorporated into chromatin covering the *GAL10* ORF. This incorporation was not directly affected by a disruption of the function of any known histone chaperone, except an indirect effect of a mutation of *SPT6*. Chromatin-bound H4 K16Ac levels were regulated by different mechanisms: At highly expressed genes, H4 K16Ac was reduced due to Spt6-mediated incorporation of K16-unacetylated histone H4, whereas at poorly expressed genes, high levels of H4 K16Ac were maintained perhaps by a RNA Pol II-mediated pass back mechanism or by histone chaperone-mediated incorporation of H4 K16Ac. We propose the existence of a nuclear pool of H4 K16Ac, which is established during S-phase and which is bound and redundantly regulated by histone chaperones, most likely Asf1 and CAF-1, and possibly Rtt106. Thus, upon functional disruption of single histone chaperones, e.g. in strains with a single deletion of *ASF1* or the CAF-1 subunit *CAC1*, no effect on H4 K16Ac enrichment upon gene repression was observed. Perhaps combinations of functional disruptions of histone chaperones would cause a significant decrease in the repression-coupled enrichment of H4 K16Ac, which would confirm a redundantly regulated pool of histone chaperone-bound H4 K16Ac. Furthermore, the results of this study as well as the fact that a deletion of *SAS2* is not lethal show that the genome-wide incorporation of Sas2-mediated H4 K16Ac during S-phase is not essential to prevent the spurious binding of SIR proteins, which would lead to silencing of euchromatic regions. Thus, it remains to be investigated, which function Sas2-mediated H4 K16Ac in euchromatin serves. In addition, it needs to be determined how SIR spreading, especially at heterochromatin-euchromatin boundaries, is prevented during G1-phase, since H4 K16Ac has been shown to become deacetylated during M-phase to promote chromatin condensation.

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6 Appendix

The HAT Sas2, which catalyzes the acetylation of lysine 16 of histone H4 (H4 K16Ac), has previously been shown to interact with the chromatin assembly factors Asf1 and CAF-1 (Meijsing and Ehrenhofer-Murray 2001). CAF-1 is mainly active during S-phase in depositing H3/H4-tetramers onto the DNA as a first step in nucleosome assembly (Smith and Stillman 1989; Kaufman et al. 1997). Both of these chromatin assembly factors interact with the sliding clamp PCNA (Huang et al. 2005). Single deletions of *ASF1* or subunits of CAF-1 (*CAC1*, *CAC2*, *CAC3*) as well as double deletions of *ASF1* in combination with a subunit of CAF-1 are not lethal in *S. cerevisiae*, but show effects on silencing at telomeres and silent mating-type loci (Tyler et al. 1999). This has led to the hypothesis, that CAF-1 as well as Asf1 predominantly assemble nucleosomes at heterochromatic loci within the yeast genome (Tyler 2002). So far, the experimental proof for this hypothesis is missing.

In order to investigate at which loci of the genome of *S. cerevisiae* CAF-1 assembles nucleosomes on newly replicated DNA the following approach was pursued. The aim of the experiments was to identify genomic loci where the largest subunit of CAF-1, Cac1, was bound compared to the localization of replication forks during a whole S-phase. This was to be addressed by generating a yeast strain with a tagged version of Cac1 and a differently tagged subunit of the replication machinery whose passage along the genome can be observed (this was done for the catalytic subunit of the DNA polymerase ε, Pol2, as well as for Cdc45). Cells of such a strain were to be arrested in G1-phase using the α-factor mating pheromone and then synchronously released into the following S-phase, taking samples of cells for chromatin immunoprecipitation against the tagged versions of Cac1 and the replication machinery subunit at defined time points after the release into S-phase. The immunoprecipitated DNA should then be sequenced (ChIP-seq) and a time-resolved, genome-wide association map of Cac1 in correlation with the localization of replication forks over a whole S-phase should be generated by appropriate data analysis. Furthermore, in a next step, a similar chromatin association map of Sas2 over a whole S-phase should be generated.

During the course of this study, different strains carrying tagged versions of the replication machinery subunits Pol2 or Cdc45 in combination with a tagged version of Cac1 were generated. Furthermore, strains with tagged versions of the replication machinery subunits Pol2 or Cdc45 from other labs (AEY5099 and AEY5120), carrying an additional deletion of *BAR1* to improve the ability of the cells to arrest in G1-phase with α-factor, were tested in ChIP experiments. Exemplarily, problems that emerged regarding the synchronization of cells

and results of the ChIP of the tagged subunits of the replication machinery at well-characterized loci are presented here.

For cell-cycle synchronization, cells were arrested with α-factor and synchronously released into S-phase by the addition of the protease pronase, which cleaves α-factor. Cell-cycle synchrony in all cells of the culture was an essential prerequisite for the ChIP experiment to work, because otherwise replication would not be synchronous, leading to diffuse peaks of chromatin association of the replication machinery at defined genomic loci. For an improved temporal resolution of the S-phase, cells were grown at 16°C. For strains with or without a deletion of *BAR1*, different amounts of α-factor for the G1-arrest and pronase for the subsequent release into S-phase were tested. Concentrations of α-factor and pronase, found to be suitable, as well as the exact synchronization protocol are presented in 2.6.

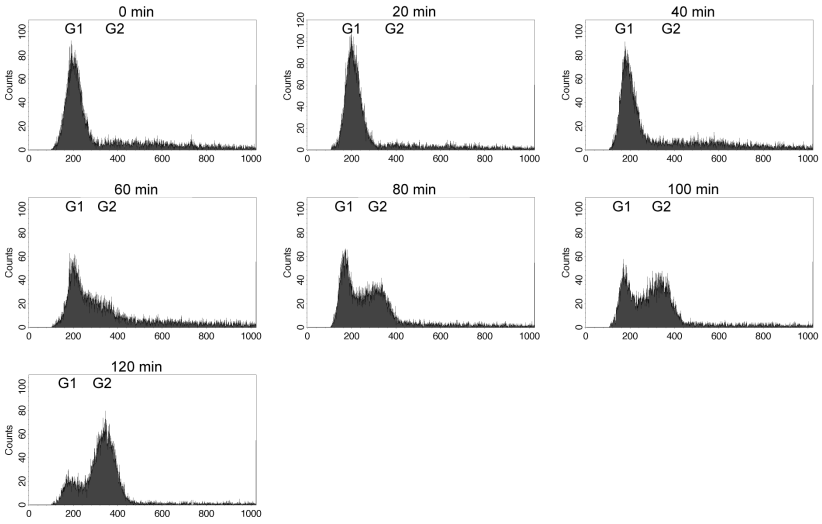

Figure 34. Cells were not synchronously released into S-phase.
Example of a cell synchronization experiment. Cells expressing a Pol2-13myc construct in *bar1*Δ background (AEY5120) were arrested in G1-phase by the addition of α- factor (f. c. 25 ng/ml) and released into the following S-phase by the addition of pronase (f. c. 100 µg/ml) at 16°C culturing temperature. Samples for the analysis of the DNA content by FACS were taken at the indicated time points. Cells were simultaneously arrest in G1-phase and also went through S-phase simultaneously until 60 min after release. A subpopulation of cells within the culture then seemed to be delayed in replication progression because a peak representing G1 DNA content remained while the G2 peak further increased. 120 min after the release, the cells had progressed through a whole S-phase.

One problem with cells progressing through S-phase is exemplarily illustrated in Figure 34. Cells were synchronously arrested in G1-phase (time point "0 min") and further progressed synchronously through S-phase until 60 min after the release into S-phase. At later time points (80 and 100 min), one subpopulation of cells within the culture seemed to be delayed, represented by the lack of a decrease of cells with a 1N DNA content (G1) while increasing the G2 peak (2N DNA content) in the FACS profiles. At 120 min after the release, all cells

had progressed through a whole S-phase. This asynchrony, which was observed in almost all synchronization experiments performed to address the movement of replication forks by ChIP, had severe effects on the results of ChIP against a tagged version of Pol2 or Cdc45. As a control, the immunoprecipitated DNA from a ChIP experiment against tagged Pol2 or Cdc45 was quantified at two well characterized, early-firing origins of replication on chromosome III (ARS305 and ARS306) and three loci between these origins of replications (Figure 35A). Initiating at ARS305 and ARS306, replication forks move towards each other. Peaks of chromatin association of replication machinery components were expected to be seen chronologically first at the origins of replication, second at loci 8 kb away from the origins and finally in the center between both origins (ARS305+17kb) (Aparicio et al. 1997).

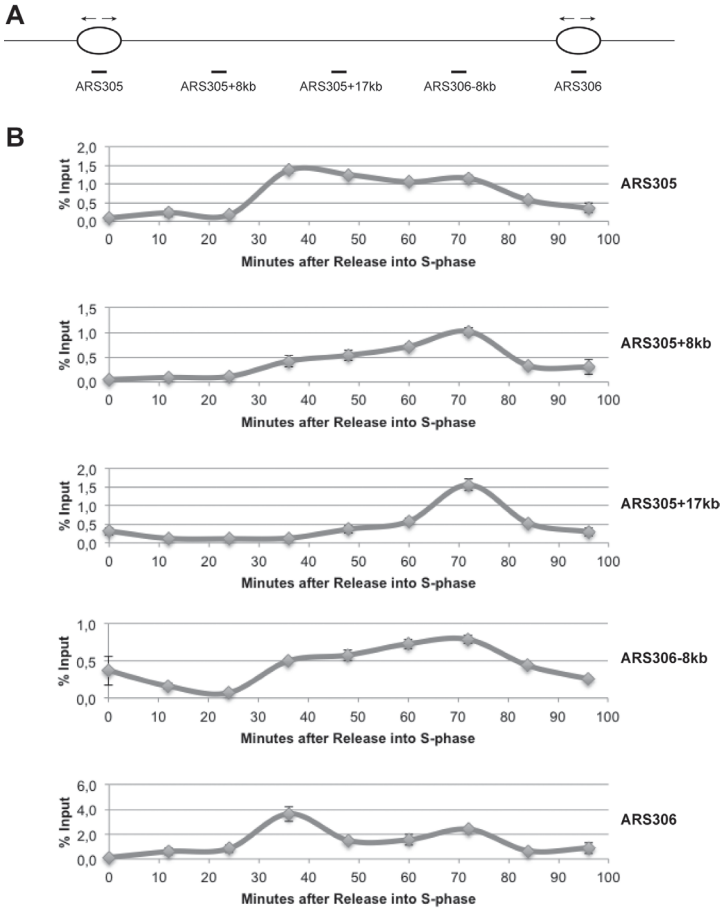

Figure 35. The movement of replication forks between ARS305 and ARS306 could not be clearly detected by ChIP for Pol2-13myc.
(A) As control, chromatin association of subunits of the replication machinery was monitored at two early-firing origins of replication (ARS305 and ARS306) and three loci between these ARS sequences. Initiating at ARS305 and ARS306, replication forks move towards each other (as marked by the arrows). Peaks of chromatin association of replication machinery components should then be seen chronologically at first at the origins of replication, secondly at loci 8 kb away from the origins and at last in the center between both origins (ARS305+17kb). (**B)** Example of a ChIP experiment with cells expressing a Pol2-13myc construct in *bar1*Δ background (AEY5120). ChIP against the myc-tag of Pol2-13myc was performed in samples taken at the indicated time points after release into S-phase. Chromatin association of Pol2-13myc was measured at the loci presented in (**A**). Pol2-13myc association to chromatin was not observed as expected: Pol2-13myc peaks did not chronologically appear at first at the origins of replication, followed by the loci 8 kb away from the ARS

sequences, followed by the locus in the middle between both ARS sequences. Instead of clear peaks of association, diffuse peaks had been observed at all five loci investigated here.

However, no clear chronological progression of replication forks initiating at the ARS305 and 306 while moving towards each other could be observed in ChIP experiments neither against tagged Pol2, nor against tagged Cdc45. In Figure 35B, chromatin enrichment of Pol2-13myc at the described loci between ARS305 and 306 in a representative experiment is shown. No clear peaks of chromatin association of Pol2-13myc at defined time points after the release into S-phase could be observed. Instead, diffuse peaks of chromatin association were seen, most likely representing the consequences of an incomplete synchronization of the cells, as shown in Figure 34. Thus, the progression of replication forks could not be traced.

Although different approaches for cell-cycle synchronization and different ChIP protocols were tested in different yeast strains, the problems with the traceability of replication fork progression could not be eliminated during the course of this study. Thus, the experiment to generate a time-resolved, genome-wide association map of Cac1 in correlation with the localization of replication forks over a whole S-phase could not be performed.

Danksagung

Mein ganz besonderer Dank gilt Ann Ehrenhofer-Murray für die Bereitstellung dieses spannenden Promotionsthemas, für die exzellente Betreuung, die vielen hilfreichen Diskussionen, die bioinformatische Auswertung von ChIP-chip-Daten und die experimentelle Unterstützung während der Anfertigung dieser Arbeit.

Ich danke Franziska Heise für ihre experimentelle Arbeit, welche die Grundlage für viele der in dieser Arbeit durchgeführten Experimente bildete. Außerdem danke ich ihr für viele hilfreiche Erklärungen insbesondere am Anfang meiner Arbeit.

Mein herzlichster Dank gilt weiterhin Allen, die mich technisch bei meiner Arbeit unterstützt haben: Ludger Klein-Hitpass für die Verarbeitung und Hybridisierung der ChIP-chip-DNA; Ho-Ryun Chung für bioinformatische Analysen; Karolin, Anke, Tanja, Gesine und Martina für ihre exzellente Hilfe bei der Durchführung von Experimenten; den Mitarbeitern der AG Ehrmann für die Zurverfügungstellung der qPCR-Maschine; Gabriele Schreiber und Christiane Müller für ihre Hilfe bei FACS-Messungen.

Ich bedanke mich bei Maria Vogelauer und Virginia Zakian, die Hefestämme für diese Arbeit zur Verfügung gestellt haben.

Allen ehemaligen und derzeitigen Kollegen aus Essen und Berlin, insbesondere auch den noch nicht genannten, Juliane, Martin, Jan, Jessica, Maria, Stefan, Christiane, Christian und Rita, sowie Josta, Laura und Ed, danke ich für die gute Zusammenarbeit, die Diskussionsbereitschaft und das angenehmen Arbeitsklima.

Weiterhin möchte ich mich bei den Organisatoren und den ehemaligen und derzeitigen Mitgliedern der Graduiertenschule BIOME an der Universität Duisburg-Essen für die vielen anregenden Diskussionen und Ratschläge bedanken.

Mein ganz besonderer Dank gilt meiner Familie für ihre immerwährende Unterstützung.

Publikationen

Reiter C, Heise F, Chung HR, Ehrenhofer-Murray AE. A link between Sas2-mediated H4 K16 acetylation, chromatin assembly in S-phase by CAF-1 and Asf1, and nucleosome assembly by Spt6 during transcription. (in Vorbereitung)

Posterpräsentationen

Reiter C, Heise F, Ehrenhofer-Murray AE. 2013. Involvement of chromatin assembly factors in Sas2-mediated H4 K16 acetylation in *Saccharomyces cerevisiae*. *Chromatin, Replication and Chromosomal Stability*, 17.-19.06.2013, Kopenhagen, Dänemark.

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Berlin, den 22.05.2014 Christian Reiter

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, den 22.05.2014 Christian Reiter